

Dear Prof. Lisanti, Editor-in-Chief,

thank you very much for the opportunity to submit a second revised version of the paper 'Autophagic degradation contributes to muscle wasting in cancer cachexia'. The paper has been modified according to the reviewer's suggestions, including the experiments on differentiated myotubes (last point raised by the reviewer), despite these were not required in the first revision. The data obtained by treating C2C12 myotubes with low TNF α concentrations (5 ng/ml), as well as those obtained by injecting the cytokine in the skeletal muscle of intact animals, have now been provided. Just a comment, in this regard: we are asked to expose C2C12 cultures to 'physiological' concentrations of TNF α , and, on the other side, to verify that this cytokine induces muscle autophagy *in vivo*. However, all the possible ways to test TNF α effects *in vivo*, including those suggested by the reviewer, require (Moresi V. et al., PLoS ONE 4:e5570, 2009), or result in (Oloff A. et al., Cell, 50:555-563, 1987; Coletti D. et al., Genesis, 43:120-128, 2005) high, markedly supraphysiological concentrations of the cytokine, that will very likely affect the whole organism and not just the treated muscle. As an example, the study by Coletti and coworkers demonstrates that TNF α gene transfer into the skeletal muscle induces systemic cachexia. In conclusion, with the due respect, we wonder about the physiological significance of the results obtained from such a study, and this was precisely the reason that lead us to discard the execution of the experiment during the first revision.

We really hope that in the present version the paper would become suitable for publication in The American Journal of Pathology.

Best regards.

Paola Costelli

Point-by-point reply

- a more accurate attempt to define the novelty of the present study in comparison with the data already available in the literature has been performed. Previous studies reporting data pertinent to the activation of autophagy in the model systems used in the present paper have been cited in the Introduction, taking care to underline the differences in terms of both approaches and results (page 4, lines 10-25) and not only in the Discussion as in the previous revised version;
- the experiments on C2C12 cultures have been repeated by using TNF α 5 ng/ml, in the presence or in the absence of bafilomycin A in order to assess also the

'autophagic flux'. The data are reported in Figure S4B and in the Result section (page 12, line 28 – page 13, line 5). Such experiments have been performed on fully differentiated myotubes (6 days after switch to differentiation medium), as requested by the reviewer, although this point was not raised in the first revision;

- an *in vivo* experiment has been performed to investigate if TNF α directly induces autophagy in the skeletal muscle. According to the reviewer's suggestion, we injected the cytokine in the gastrocnemius muscle of healthy mice. The results are shown in Figure S5 and discussed in the text (page 13, lines 14-19). TNF α concentration (40 μ g/kg body weight) has been selected according to the data available in the literature (Langen et al., FASEB J, 18:227-237, 2004). Untreated mice have been used as controls, in order to avoid biases due to the probable systemic effects of intramuscular TNF α injection.

AUTOPHAGIC DEGRADATION CONTRIBUTES TO MUSCLE WASTING IN CANCER CACHEXIA

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Running title: autophagy in cachexia

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ABSTRACT

Muscle protein wasting in cancer cachexia is a critical problem. The underlying mechanisms are still unclear, although the ubiquitin-proteasome system has been involved in the degradation of bulk myofibrillar proteins. The present work has been aimed to investigate if also autophagic degradation plays a role in the onset of muscle depletion in cancer-bearing animals as well as in glucocorticoid-induced atrophy and sarcopenia of aging. The results show that autophagy is induced in muscle in three different models of cancer cachexia as well as in glucocorticoid-treated mice. By contrast, autophagic degradation in the muscle of sarcopenic animals is impaired, but can be reactivated by calorie restriction. These results further demonstrate that different mechanisms are involved in pathological muscle wasting, and that autophagy, either excessive or defective, contributes to the complicated network that leads to muscle atrophy. In this regard, particularly intriguing is the observation that in both cancer hosts and TNF α -treated C2C12 myotubes insulin can only partially blunt autophagy induction. This would suggest that autophagy is triggered through mechanisms that cannot be circumvented by using classical up-stream modulators, prompting to identify more effective approaches to target this proteolytic system.

1 INTRODUCTION

2 Cancer cachexia is a multifactorial syndrome characterized by anorexia, weight loss and
3 muscle wasting that significantly impairs patients' quality of life and survival, also reducing their
4 tolerance to anti-neoplastic treatments. Despite the relevance of cachexia to patient outcome,
5 however, effective cachexia treatments are still lacking, and only recently tumor-induced wasting
6 is becoming to be considered as a prognostic tool ¹.

7 The mechanisms underlying muscle wasting in cancer cachexia are still unclear, although
8 experimental and clinical studies have shown that hyperactivation of the ubiquitin-proteasome-
9 dependent proteolytic system plays a critical role ^{2,3}. In this regard, however, in the last few years
10 the involvement of autophagic-lysosomal proteolysis has also been proposed. The lysosomal
11 degradative system contributes to different cellular processes, such as degradation of cytoplasmic
12 proteins and organelles, limited cleavage or extensive degradation of various substrates,
13 trafficking and recycling of molecules among internal organelles to and from the exterior of the
14 cell, post-translational maturation of secretory products and storage of undigested material ⁴.
15 Autophagy is the major process for degradation of cellular constituents, its rate being enhanced
16 under stress conditions leading to organelle damage or under marked nutrients restriction
17 (starvation), in order to recycle biomolecules for the synthesis of essential constituents ⁵. In such
18 conditions, autophagy has been shown to operate bulk proteolysis in most visceral tissues, such as
19 the liver ⁶, while its role in the skeletal muscle has been emerging only during the last years. In this
20 regard, few years ago a study reported for the first time that starvation-induced autophagy also
21 occurs in the skeletal muscle ⁷, and quite recently autophagy has been shown to contribute to
22 starvation-induced muscle atrophy, with a mechanism involving TNF α receptor-associated factor
23 (TRAF)6/Fn14 signaling ⁸. An altered lysosomal function has also been reported in several
24 myopathies ⁹ and, more recently, autophagy-related genes (ATGs) have been shown to be
25 induced in muscle by denervation or fasting, through a FoxO3-dependent mechanism ¹⁰. In this
26 regard, FoxO3 has been proposed to regulate both autophagy and proteasome-dependent
27 proteolysis ¹⁰. However, a sort of hierarchy appears to exist between these two processes, since a
28 parallel study shows that autophagic degradation induced by starvation or FoxO3 overexpression
29 is sufficient to determine muscle depletion even if the ubiquitin-proteasome degradation is
30 blocked using pharmacological or genetic approaches ¹¹.

As for cancer cachexia, results obtained on muscles isolated from cachectic animals led to rule out a substantial role for lysosomes in the overall protein degradation¹². On the other hand, an elevation of total lysosomal protease activity has been observed in the skeletal muscle and liver of tumor-bearing rats¹³, and muscle depletion could be prevented by treatment of tumor hosts with leupeptin, an inhibitor of cysteine proteases such as cathepsins B, H, and L¹⁴. Finally, increased levels of cathepsin L mRNA have been reported in the skeletal muscle of septic or tumor-bearing rats, while cathepsin B gene expression has been shown to be enhanced in muscle biopsies obtained from lung cancer patients^{15, 16}.

The present work has been aimed to investigate the relevance of autophagy to the pathogenesis of skeletal muscle wasting in cancer cachexia. In this regard, only recently few and scanty observations suggested that autophagy can be activated in the muscle of animals bearing the Lewis lung carcinoma or the colon 26 tumor^{8, 17-19}. However, a comprehensive analysis of the activation of the autophagic-lysosomal proteolytic pathway, including the evaluation of autophagic flux, in cancer hosts is still lacking. The present study fills this gap. Not only, cancer-induced muscle wasting has been compared, in terms of activation of autophagy, with that occurring in other experimental conditions such as glucocorticoid-induced atrophy and aging. On this line, previous data reported that autophagy significantly contributes to muscle wasting in different experimental conditions, such as denervation, starvation and sepsis^{10, 20}. However, while some of the mechanisms underlying glucocorticoid-induced atrophy have already been described²¹⁻²³, only two reports have provided little evidence, also indirect, about the involvement of autophagy^{24, 25}. The present study, extensively investigating the effects of glucocorticoid administration on the activation of muscle autophagy, clarifies this point. As for aging, only a couple of reports^{26, 27} analyzed the modulations of muscle autophagy. The present study extends such findings providing a panel of autophagy markers in the aged muscle at protein levels, and comparing their changes with those occurring in aged animals exposed to 40% calorie restriction.

The results show that autophagic-lysosomal degradation is induced in three different models of cancer-associated muscle atrophy as well as in glucocorticoid-treated animals, suggesting this proteolytic system as a possible target of therapeutic strategies.

1 MATERIALS AND METHODS

2 All materials were supplied by Sigma-Aldrich, unless differently specified.

3 Animals and experimental design

4 Experimental animals were cared for in compliance with the Italian Ministry of Health
5 Guidelines (n° 86609 EEC, permit number 106/2007-B) and the Policy on Humane Care and Use of
6 Laboratory Animals (NIH 1996). The experimental protocol was approved by the Bioethical
7 Committee of the University of Torino. Male Wistar rats weighing about 150 g, Balb-c and
8 C57/BL6 mice weighing about 20 g were obtained from Charles River Laboratories, and maintained
9 on a regular dark-light cycle (light from 08:00 to 20:00), with free access to food and water during
10 the whole experimental period. Tumor-bearing rats (n = 8) received an intraperitoneal (i.p.)
11 inoculum of Yoshida AH-130 ascites hepatoma cells (~10⁸ cells/rat; provided many years ago by
12 Prof. M. Del Monte, University of Milano, Italy, and maintained in our lab by weekly i.p.
13 transplantation), while tumor-bearing mice (n = 8) were inoculated subcutaneously dorsally with
14 5x10⁵ C26 carcinoma cells (kindly provided by Prof. M.P. Colombo, IRCCS National Cancer
15 Institute, Milano, Italy) or intramuscularly with 5x10⁵ Lewis Lung carcinoma cells (ATCC, USA).
16 Both C26 and LLC cells were expanded in vitro (50.000 cm², Dulbecco's Modified Eagle's Medium
17 (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml
18 sodium pyruvate, 2 mM L-glutamine, maintained at 37°C in a humidified atmosphere of 5% CO₂ in
19 air), detached with trypsin, re-suspended in sterile saline and subsequently implanted in the
20 animals at the concentrations indicated above. Rats or mice inoculated with vehicle (saline) served
21 as controls (n = 6). In another set of experiments, animals were randomized and divided into four
22 groups, namely controls (C, n = 6) and C26 (n = 8), treated or not with insulin (INS). INS groups
23 received daily i.p. injections of insulin (1,5 IU/kg b.w.) during the whole experimental period. For
24 'autophagic flux' experiments controls (C, n = 6) and C26 (n = 6) mice were either administered
25 colchicine 0,4 mg/kg/day or vehicle for two days before sacrifice (both day 11 and day 14 of tumor
26 growth). Dexamethasone (Dexa) was administered i.p. daily for 7 days to 6 week old Balb-c mice
27 (n=8) at the dose of 1 mg/kg. Vehicle (saline containing 5% ethanol)-treated mice served as
28 controls (n=6). Animal weight and food intake were recorded daily. Tumor-bearing rats and mice
29 were sacrificed under anesthesia 7 and 14 days after tumor transplantation, respectively. Several
30 muscles and organs were rapidly excised, weighed, frozen in isopentane cooled with liquid
31 nitrogen and stored at -80°C. As for calorie restriction (CR), 6 month-old rats were either fed ad-

libitum (AL) or given 60% (w/w; 40% CR) of the food consumed by AL animals until 24 months of age. As for the *in vivo* TNF administration, C57/BL6 mice weighing about 20 g received an intramuscular injection of TNF as previously reported (40µg/kg body weight^{28, 29}) in the right gastrocnemius and were sacrificed after 12h. Saline treated mice in place of using the contralateral muscle served as control in order to exclude TNF-dependent systemic effects.

Insulin Tolerance Test

Control, C26 and C26 INS-treated mice at day 11 of tumor growth were challenged with insulin (1,5 IU/kg b.w., i.p.). A blood drop was collected by saphenous vein puncture before, 15, and 45 min. after insulin administration and glycemia was measured using the Glucocard G-sensor strips and apparatus (Menarini Diagnostics). Glycemia was also assessed before sacrifice collecting the blood by cardiac puncture from anesthetized animals in order to assess the relationship between glycemia and body weight changes. Correlation was calculated according to Pearson's.

Cell cultures

Murine C2C12 skeletal myoblasts (ATCC) were grown in high glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml sodium pyruvate, 2 mM L-glutamine, and maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. Differentiation was induced by shifting sub-confluent cultures to DMEM supplemented with 2% horse serum (differentiation medium; DM). At the same time, the cells were exposed to TNFα (100 ng/ml) or to INS (10ug/ml), or both, and collected after 48 h; untreated cells served as control. Such differentiation scheme was chosen after a preliminary screening of the TNFα-dependent autophagy induction in growing myoblasts, differentiating (2 days) or fully differentiated (6 days) myotubes. The differentiating cells showed the strongest autophagy alterations (data not shown) and were adopted for the following experiments. In a different set of experiments TNFα action was also tested at a lower dose (5 ng/ml) in fully differentiated myotubes. For 'autophagic flux' measurements, 200 nM Bafilomycin A1 was added 6 hours before the termination of the experiments, as previously reported³⁰. As for the data showed in Figure S6, at the time of shifting sub-confluent cultures to differentiation medium, the cells were exposed to a stable IGF-1 synthetic peptide (LONG-R3IGF-I, Sigma 85580C, 50 ng/ml) or to neutralizing anti-IGF-1 antibody (Sigma I8773), in the presence or in the absence of TNFα (100 ng/ml) and collected after 48 h; untreated cells served as control.

Primary satellite cells

Single muscle fibers with associated satellite cells were isolated from the tibialis anterior muscles after digestion with collagenase. Single myofibers were plated on matrigel-coated dishes in DMEM supplemented with 20% FBS, 10% horse serum, 0.5% chick embryo extract and penicillin-streptomycin. Three days later the medium was replaced with proliferation medium (DMEM 20% FBS, 10% horse serum, 1% chick embryo extract) to promote proliferation of detached cells (delaminated satellite cells). After 5 days (only satellite cells remain on the plate), the medium was replaced with differentiation medium (2% horse serum and 0.5% chick embryo extract in DMEM).

Western blotting

About 50 mg of gastrocnemius muscle were homogenized in 80 mM Tris-HCl, pH 6.8, containing 100 mM DTT, 70 mM SDS, and 1 mM glycerol, with freshly added protease and phosphatase inhibitor cocktails, kept on ice for 30 min, centrifuged at 15000 x g for 10 min at 4°C, and the supernatant collected. Protein concentration was assayed using BSA as working standard. C2C12 cells were lysed on RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM PMSF) with freshly added protease and phosphatase inhibitor cocktails. Equal amounts of protein (30 µg) were heat-denatured in sample-loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The filters were blocked with Tris-buffered saline (TBS) containing 0.05% Tween and 5% non-fat dry milk and then incubated overnight with antibodies directed against: Beclin1 (Sigma B6186), LC3B (Sigma L7583), p62 (BD Biosciences 610832), p-Akt (Ser473; Cell Signaling Technology 9271), atrogin-1 (ECMbiosciences AP2041) and tubulin (Sigma T5168). Peroxidase-conjugated IgG (Bio-Rad) was used as secondary antibodies. Quantification of the bands was performed by densitometric analysis using a specific software (TotalLab, NonLinear Dynamics, Newcastle upon Tyne, UK).

Reverse transcription-PCR

Total RNA was obtained using the TriPure reagent (Roche) following manufacturer's instructions. RNA concentration was determined fluorometrically using the Ribogreen reagent (Invitrogen). RNA integrity was checked by electrophoresis on 1.2% agarose gel, containing morpholino propane-sulfonic acid (MOPS) 0.02 M and 18% formaldehyde. Total mRNA was retro-transcribed using the i-Script cDNA synthesis kit (Bio-Rad). Transcript levels were determined by real-time PCR using the SsoFast Evagreen Supermix and the MiniOpticon thermal cycler (Bio-Rad).

Primer sequences were as follows: Atrogin1 FW-agcgacctcagcagttactgc, RW-cttctggaatccaggatggc; Beclin1 FW-tgaatgaggatgacagtgagca, RW-cacctggttctccacactcttg; Cathepsin L FW-gtggactgttctcagctcaag, RW-tccgtccttcgcttcatagg; LC3B FW-cactgctctgtcttgttaggttg, RW-tcgttgtgcctttattagtgcac; p62 FW-cccagtgcttggcattctt, RW-agggaaagcagaggaagctc; NBR1 FW-ccccagattgggttacaagc, RW-tccaccggttccttaaccac.

Cathepsin enzymatic activity

Cathepsin B+L activity was determined by evaluating the cleavage of a specific chromogenic substrate (see 50). 50 mg of gastrocnemius muscle were homogenized in 0,25 M sucrose, 25 mM Hepes pH 7.4, 2 mM EDTA, and then centrifuged at 2000 g for 5 min at 4 °C. The supernatant was collected and centrifuged at 70000 g for 30 min at 4 °C. The pellet was suspended in 0,1 M Na-acetate pH 5.5, 1 mM EDTA. Aliquots of 10 μ g protein were incubated for 60 min at 37 °C in the presence of 100 μ M substrate (Z-Phe-Arg-p-Nitroanilide, Enzo Life Sciences). The incubation buffer was 0,5 M Na-acetate pH 5.5, 5 mM DTT, 2,5 mM EDTA. Absorbance was read with a spectrophotometer at 410 nm (Perkin-Elmer) using free p-Nitroanilide as working standard.

Immunofluorescence

C2C12 monolayers were washed with PBS and fixed in acetone-methanol (1:1). Samples were then probed with the following primary antibodies: LC3B (Sigma L7543), p62 (BD Biosciences 610832). Detection was performed using a FITC-conjugated rabbit IgG secondary antibody. Nuclei were stained with the Hoechst 33342 fluorochrome and the images captured in an epiilluminated fluorescence microscope (Axiovert 35, Zeiss).

Data analysis and presentation

All results were expressed as mean \pm SD, with the exception of gene expression (mean \pm SEM). Representative western blots show independent samples. Significance of the differences was evaluated by analysis of variance (ANOVA) followed by Tukey's test.

RESULTS

- Autophagy is induced in C26-bearing mice

The C26 colon carcinoma induces progressive loss of body and muscle weight (see Supplemental Figure S1A and S1B at <http://ajp.amjpathol.org>). Muscle expression of proteins accepted as markers of autophagy has been assessed in the gastrocnemius muscle at both days 11 and 14 of tumor growth, representing initial and advanced stage of muscle wasting, respectively. Beclin-1, a main upstream regulator of autophagic sequestration³¹, is markedly increased already at day 11, suggesting that autophagy activation is an early event in tumor-induced muscle depletion (Fig. 1A). Secondly, levels of microtubule-associated protein 1 light chain 3B isoform I (LC3B-I) do not change in tumor-bearing mice, while the lipidated form (LC3B-II), a reliable marker of autophagosome formation³⁰, is significantly elevated at both experimental time points (Fig. 1A). Moreover, the levels of p62/SQSTM1 have been assayed as a measure of substrate sequestration into autophagosomes; p62, indeed, binds LC3 as well as substrates marked for degradation by ubiquitylation³⁰. Similarly to beclin-1 and LC3B-II, p62 accumulates at both experimental points (Fig. 1A), which could reflect either induction of autophagic sequestration or reduced autophagosome clearance³². To clarify this point, a flux experiment was performed by treating both controls and C26 hosts with colchicine, a microtubule-destabilizing agent that interacts with tubulin³³. The results show that LC3B-II levels, already higher than in controls in the C26 hosts, further increase after colchicine administration (Fig. 1B), demonstrating that autophagy is indeed activated. The reduction of tubulin expression in colchicine-treated animals (Fig. 1B) vs. untreated mice likely reflects specific drug-induced degradation³⁴, demonstrating that the treatment protocol adopted leads to bioactive colchicine levels in the skeletal muscle. Despite the flux experiment was designed in order to achieve data for both days 11 and 14 of tumor growth, only the first time-point could be evaluated; indeed, tumor-bearing animals administered colchicine on days 12 and 13 did not reach day 14 and died before sacrifice.

The possibility that also reduced autophagosome clearance might occur in the present experimental setting cannot be discarded, however. A gene study shows that p62 transcript is increased at day 14 of tumor growth (Fig. 2A), while the enzymatic activity of lysosomal cathepsins is reduced at day 14, though not at day 11 (Fig. 2B), suggesting that p62 accumulation may result from transcriptional induction and, possibly, reduced degradation. The gene study also gives evidence for increased levels of mRNAs coding for atrogin-1, cathepsin L, LC3B and NBR1, a

protein with functional similarity to p62 (Fig. 2A), further supporting the occurrence of a sustained induction of autophagy. Notably, autophagy induction in C26-bearing mice is not a feature specific for the skeletal muscle, being also detectable in the liver, wherein the pattern of LC3B and p62 expression is comparable to that observed in the gastrocnemius (see Supplemental Figure S2A at <http://ajp.amjpathol.org>).

- Autophagy is not a specific feature of the C26 hosts

To assess if the enhanced autophagic degradation is peculiar to C26-bearing mice or also occurs in other experimental models of cancer cachexia, autophagic markers have been evaluated in the gastrocnemius muscle of rats bearing the Yoshida AH-130 hepatoma or of mice transplanted with the Lewis lung carcinoma (LLC). As for the former, muscle depletion induced by 7 days of tumor growth (Fig. 3A) is associated with both increased LC3B lipidation (LC3B-II) and p62 accumulation, while no changes in beclin-1 levels occur (Fig. 3B). On the other side, muscle wasting in LLC hosts (Fig. 3C) is characterized by up-regulation of beclin-1 and p62 (Fig. 3D).

These observations indicate that, though with different pattern, autophagy is activated in all the experimental models of cancer cachexia analyzed. In this regard, these results provide the first comprehensive approach to the contribution of autophagy to the pathogenesis of skeletal muscle wasting in cancer cachexia, confirming but significantly extending previous observations (see Introduction and Discussion). In addition, these results support the idea that a single marker is not sufficient to depict the state of activation of the autophagic-lysosomal degradative system³⁰.

- Activation of autophagy in cancer-unrelated muscle wasting

To investigate whether the modulations of autophagy in cancer-related muscle atrophy are comparable to those observed in other conditions associated with muscle wasting, autophagic markers were analyzed in dexamethasone (Dexa)-induced muscle atrophy and in aging-related sarcopenia.

Dexamethasone Mice received daily i.p. injections of Dexa, a synthetic glucocorticoid whose catabolic action has been thoroughly investigated²³. Body and muscle weight loss induced by Dexa administration (7 days; Fig. 4A) are associated with increased expression of beclin-1 and both LC3B isoforms, while p62 virtually disappears (Fig. 4B). The different pattern of p62 expression in Dexa-treated mice compared to that observed in tumor-bearing animals is associated with lack of p62 transcriptional induction (Fig. 4C) as well as with normal cathepsin enzymatic activity (Fig. 4D). The gene study also shows an increased expression of atrogin-1, cathepsin L and NBR1 (Fig. 4C).

1 These observations are in line with previous data reporting that autophagy significantly
2 contributes to muscle wasting in different experimental conditions, such as denervation,
3 starvation and sepsis^{10, 20}. Not only, while providing a panel of autophagy markers (at both gene
4 and protein levels), the present results show that an efficient autophagosome clearance is
5 achieved in the muscle of glucocorticoid-treated animals, as inferred by the markedly reduced p62
6 levels.

7 Aging Both cancer and glucocorticoids induce muscle depletion in the short period (7-14 days,
8 depending on the model), and can thus be considered as 'acute' wasting conditions. On the
9 contrary, sarcopenia associated with aging is a chronic state and the underlying mechanisms could
10 be different. To test the relevance of autophagy to sarcopenia, the same markers reported above
11 have been measured in aged mice and rats. In 22 month old mice both beclin-1 and LC3B-I protein
12 levels fall, while p62 markedly accumulates in comparison with 3 month old mice (Fig. 5A),
13 suggesting a reduced autophagic degradation. As for the rats, the comparison between 6 and 24
14 month old animals shows the accumulation of p62 (Fig. 5B). In the attempt to stimulate the
15 autophagic flux, rats have been submitted to 40% calorie restriction, an anti-aging strategy
16 previously shown to activate liver autophagy³⁵. In the present conditions, calorie restriction
17 reduces both LC3B-II and p62 levels in the skeletal muscle, likely releasing the autophagy blockade
18 (Fig. 5B).

19 - **Autophagy in cancer-induced muscle atrophy is only partially modulated by insulin**

20 Autophagy is mainly regulated by the PI3K/Akt/mTOR (mammalian Target of rapamycin)
21 pathway and previous data showed that insulin administration effectively prevented muscle
22 protein hypercatabolism in rats bearing the AH-130 hepatoma¹⁴. To test if insulin effectiveness in
23 preventing muscle wasting could rely on down-regulation of the autophagic degradation, the
24 hormone was administered to C26-bearing mice, the model system where autophagy appears
25 more marked. In contrast with the results previously obtained in the AH-130 hosts¹⁴, insulin
26 administration does not attenuate cachexia in the C26 hosts (Fig. 6A). Beclin-1 and p62 protein
27 levels in insulin-treated tumor hosts are reduced in comparison to the untreated animals, but
28 remain significantly higher than in controls, demonstrating that insulin inhibition of autophagy is
29 far from being complete (Fig. 6B). Such a pattern is not peculiar to the skeletal muscle, since
30 comparable results can be observed in the liver (see Supplemental Figure S2B at
31 <http://ajp.amjpathol.org>). The gene study analysis shows that atrogin-1, beclin-1 and p62

1 transcripts are reduced by insulin, while cathepsin L and LC3B are even increased (Fig. 6C). The
2 partial restoration of physiological autophagy exerted by insulin could reflect a condition of mild
3 resistance to this hormone. In this regard, a study reported in the literature suggests that muscle
4 wasting in C26 hosts is associated with insulin resistance¹⁷. To clarify this point, an insulin
5 tolerance test has been performed. The results show that both control and tumor-bearing animals,
6 either treated with insulin or not, respond comparably to insulin challenge, thus excluding the
7 occurrence of insulin resistance (see Supplemental Figure S3B at <http://ajp.amjpathol.org>). In
8 addition, the direct correlation existing between basal glycemia and body weight changes over the
9 experimental period (see Supplemental Figure S3C at <http://ajp.amjpathol.org>), suggests that, at
10 least in this experimental setting, hypoglycemia rather than insulin resistance contributes to the
11 loss of body weight.

12 - **TNF α directly stimulates autophagic degradation in skeletal muscle cell cultures.**

13 Muscle wasting in cancer cachexia is largely mediated by proinflammatory cytokines. Indeed,
14 protein hypercatabolism can be partially reversed by treating rats bearing the AH-130 hepatoma
15 with anti-TNF α antibodies or with pentoxifylline, an inhibitor of TNF α synthesis^{36,37}. We examined
16 whether TNF α has the ability to directly activate the autophagic process in differentiating C2C12
17 myocytes cultured in the presence of the cytokine for 48 hours. Similarly to a previous study
18 obtained with the cytokine TWEAK (TNF-like weak inducer of apoptosis)³⁸, TNF α causes a
19 reduction of Akt phosphorylation (Fig. 7A). This observation seems in contrast with previous data
20 showing increased levels of phosphorylated Akt in TNF α -treated C2C12 myotubes³⁹; the
21 discrepancy may well result from the fact that the experiments here shown were performed on
22 differentiating C2C12 myocytes (see Materials and Methods). The reduced levels of active Akt are
23 associated with beclin-1 induction and LC3B-II accumulation (Fig. 7A), in the absence of significant
24 changes of atrogin-1 expression ($C = 0.55 \pm 0.15$, $TNF = 0.39 \pm 0.15$, a. u., $n = 3$). Activation of
25 autophagy is also suggested by the increased p62 (Fig. 7B) and dotted LC3B staining ($C = 4 \pm 2$, TNF
26 $= 41 \pm 9$, cells with more than 10 puncta/60 nuclei, $n = 3$, $p = 0.0024$; Fig. 8B). A comparable
27 pattern can be observed also in primary satellite cells exposed to TNF α (see Supplemental Figure
28 S4A at <http://ajp.amjpathol.org>), as well as in C2C12 myotubes exposed to low TNF α
29 concentrations (5 ng/ml; see Supplemental Figure S4B at <http://ajp.amjpathol.org>). Finally,
30 'autophagic flux' was evaluated by incubating C2C12 cultures with bafilomycin A1, a drug able to
31 inhibit the vacuolar H⁺-ATPase, resulting in reduced acidification and, therefore, lysosomal-

1 dependent degradation of the autophagosome cargo³³. The results show that LC3B-II levels in
2 bafilomycin A1-treated cells markedly increase with respect to controls. When cultures are
3 exposed to TNF α and bafilomycin A1, LC3B-II levels are significantly higher than those measured in
4 cells treated with bafilomycin A1 alone (Fig. 8A, see Supplemental Figure S4B at
5 <http://ajp.amjpathol.org>). This observation suggests that the rate of LC3B-II formation is increased
6 by the cytokine (see⁴⁰). Conversely, LC3B-II levels in C2C12 cultures treated with TNF α alone (100
7 ng/ml, Fig. 8A) are not different from those detected in cells exposed to TNF α and bafilomycin A1.
8 Such an observation would suggest that TNF α inhibits autophagosome degradation rather than
9 enhancing their formation. However, lysosomal proteolysis should be completely blocked by
10 bafilomycin; on this line, LC3B-II increase over bafilomycin-induced levels should result from
11 enhanced autophagosome formation⁴⁰. This conclusion is strengthened by the results obtained on
12 C2C12 myotubes exposed to TNF α 5 ng/ml (Fig. S4B). In this regard, the results obtained on C2C12
13 cells are on the same line of those reported for the muscle of the C26 hosts, where both inhibition
14 of autophagosome degradation and increased sequestration are likely to occur. To ascertain if
15 autophagy can be activated by TNF α also in vivo, healthy mice received an intramuscular injection
16 of this cytokine (40 μ g/kg body weight^{28, 29}) and were sacrificed 12 h later. Figure S5 (available at
17 <http://ajp.amjpathol.org>) shows that LC3B-II levels are increased in TNF α -treated muscles in
18 comparison to the muscle of control mice, the more so when the animals also received colchicine,
19 indicating the enhancement of autophagic flux.

20 The data reported above indicate that exposure of muscle cell cultures to TNF α results in
21 upregulation of autophagy. To clarify if this effect is responsive to anabolic regulation, C2C12 cells
22 were cotreated with TNF α and insulin. As expected, the results show that insulin alone increases
23 Akt phosphorylation (C = 0.21 \pm 0.05, INS = 0.31 \pm 0.03, a. u., n = 3, p<0.05) and reduces both
24 atrogin-1 expression (C = 0.55 \pm 0.15, INS = 0.28 \pm 0.05, a. u., n = 3, p<0.05) and LC3B-II levels (Fig.
25 7A). These observations, together with the enhanced immunostaining for p62 (Fig. 7B), suggest
26 that basal autophagy is inhibited. When insulin is coupled to TNF α , no significant changes could be
27 observed as for atrogin-1 expression (INS = 0.28 \pm 0.05, INS+TNF = 0.24 \pm 0.06, a. u., n = 3) and Akt
28 phosphorylation (INS = 0.31 \pm 0.03, INS+TNF = 0.18 \pm 0.10, a. u., n = 3; Fig. 7A), although a tendency
29 to reduction is apparent. Despite insulin exposure, both TNF α -induced beclin-1 and LC3B-II
30 increase remain detectable (Fig. 7A). In the presence of bafilomycin A1, insulin results in LC3B-II
31 accumulation lower than in cells exposed to the lysosomal inhibitor alone (Fig. 8B). However, in

1 insulin and bafilomycin A1 treated cultures, LC3B-II levels in the presence of TNF α are higher than
2 those observed in the absence of the cytokine, although they do not reach those obtained in cells
3 exposed to bafilomycin A1 alone (Fig. 8B). Such observation suggests that insulin reduces basal
4 autophagic flux but is unable to completely prevent the activation induced by TNF α exposure. This
5 hypothesis is confirmed by LC3B immunostaining (INS = 5 ± 1 , TNF+INS = 60 ± 9 , cells with more
6 than 10 puncta/60 nuclei, n = 3, p = 0.00025; Fig. 7B). Similar results have been obtained by
7 treating the cells with recombinant stable IGF1, while treatment with an IGF1 neutralizing
8 antibody is sufficient to activate autophagy, although to levels lower than those attained by TNF α
9 (see Supplemental Figure S6A and S6B at <http://ajp.amjpathol.org>).
10

DISCUSSION

The present study demonstrates that muscle wasting in three different experimental models of cancer cachexia is associated with increased autophagy, as evaluated by analyzing three among the most representative markers of this degradative pathway: beclin-1 as an indicator of autophagy induction, LC3B conversion to the lipidated form to measure autophagosome abundance and p62/SQSTM1 as a marker of substrate sequestration and eventual degradation (see Results). The analysis of these proteins gives an approximate estimate of the autophagic flux, i.e. the extent of protein sequestration and subsequent degradation into lysosomes. To further strengthen the data, autophagic flux was measured by treating C26 hosts with colchicine (see ³³). Although the markers of autophagy mentioned above do not strictly share the same pattern of expression in the cachexia models used in the present study, the net result is that in all of them an increase of autophagic degradation is likely to occur. This observation represents an important new finding. Indeed, while muscle wasting in cancer cachexia is considered to depend mainly on hyperactivation of proteasomal degradation ^{2, 41-44}, only recently beclin-1 and LC3B induction was shown in LLC-bearing mice ⁸, while Bnip3 overexpression or increased LC3B-II levels in the C26 hosts were previously reported ^{17, 18}. Finally, autophagy was proposed to contribute to C26-induced cardiac atrophy ¹⁹.

To the best of our knowledge, the data shown in the present study represent the most comprehensive analysis actually available showing how the autophagic system is poised towards activation in the skeletal muscle of the C26-bearing mice. Similar conclusions can be drawn from results obtained in rats bearing the AH-130 Yoshida hepatoma, where hyperactivation of muscle lysosomal proteases was already shown by Tessitore et al. ¹³, and in mice implanted with the LLC tumor, these latter confirming but extending what previously reported by Paul and coworkers ⁸. These results highlight the relevance of autophagy to cancer-induced muscle wasting, the more so when considering that not only the upstream signals that activate protein breakdown are able to coordinate both autophagy and proteasome induction ^{45, 46}, but also that typical proteasome substrates (e.g. ubiquitylated proteins) can be alternatively degraded through the acidic vacuolar proteolysis ⁴⁷.

A careful analysis of the results here presented suggests that, despite autophagy is activated in both cancer cachexia and dexametasone-induced muscle atrophy, but not in sarcopenic muscles, different mechanisms are operating in the specific experimental conditions.

As for cancer cachexia, all the markers of autophagy are up-regulated in the C26 hosts. Particularly interesting in this regard is the marked accumulation of p62, that contrasts with the observation that autophagic flux is clearly enhanced, at least in d11 C26 hosts (see Fig. 1B). Such increased p62 levels could reflect a transcriptional induction, as reported in desmin-related cardiomyopathy⁴⁸. Consistently, p62 gene expression is markedly increased in the muscle of the C26 hosts. Autophagic flux measurement was not possible in day 14 C26 hosts, since colchicine-treated animals died before sacrifice, suggesting that autophagy inhibition negatively impinges on survival when the metabolic homeostasis is markedly compromised.

Despite the induction of autophagy, muscle lysosomal cathepsin activities are reduced in day 14 C26 bearers, a finding that may reflect selective disturbances in lysosomal function, such as lysosomal enzyme mistargeting⁴⁹. In this regard, the possibility that during tumor growth muscle protein degradation relies initially on the lysosomal system and only subsequently on other proteolytic pathways cannot be excluded. Consistently, lysosomal protease activity is reduced only in day 14 C26 hosts (advanced cachexia), in parallel with both increased atrogin-1 mRNA levels and calpastatin degradation, this latter being a marker of calpain hyperactivation (unpublished data).

As for dexamethasone-induced atrophy, the results show that both beclin-1 and LC3B-II increase, while p62 virtually disappears, indicating that substrates sequestered into autophagosomes are readily degraded by lysosomes. The lack of p62 accumulation in the skeletal muscle of dexamethasone-treated mice is associated with unchanged levels of p62 transcript. In addition, this observation suggests that even if rapid muscle atrophy observed in both tumor bearers and glucocorticoid-treated mice is characterized by autophagic degradation, the molecular signals involved are different and may require distinct therapeutic approaches.

Finally, autophagic degradation is reduced in the skeletal muscle of aged animals while is reactivated by CR, consistently with recent data²⁶. Reduced muscle mass occurs in both aging and CR⁵⁰, however the pattern of the autophagic markers is quite different. Indeed, while in aged animals both beclin-1 and LC3B-II are comparable, or even reduced vs. young individuals, p62 levels are markedly increased, likely because autophagy is not activated, and waste substrates accumulate. While impairment of autophagic degradation during aging is a well known event in the liver, the present observations represent a new finding, since very few data are actually available as for the skeletal muscle²⁶. In this regard, abnormal mitochondria and sarcomere disorganization in aged skeletal muscle likely results from impaired autophagy. Consistently,

muscle-specific deletion of Atg7, a crucial autophagy gene, leads to phenotypic alterations resembling sarcopenia⁵¹. As for CR, it is associated with p62 levels that are still increased with respect to young animals, but markedly reduced in comparison to aged rats, suggesting a partial restoration of the autophagic flux. These observations are in agreement with a previous report by Wohlgemuth et al.²⁶, showing that 8% calorie restriction was able to partially restore the reduced expression of autophagic markers in aged rats.

In the C26 host, energy deficit, whose occurrence is suggested by the correlation between hypoglycemia and body weight loss as well as by mitochondrial damage (Penna et al., unpublished data), is a possible trigger of autophagy, not only in the skeletal muscle, but also in the liver⁵². Such a speculation would fit with the recently proposed model of “battery-operated tumor growth”, where autophagic degradation is suggested to start in the tumor stroma and then to spread systemically in order to provide a continuous energy transfer from the host to the tumor⁵³.

The onset of cancer cachexia is also associated with a proinflammatory environment^{54, 55}. In this regard, anti-TNF α strategies have been shown to effectively prevent the hyperactivation of proteasomal degradation in the AH-130 hosts^{36, 37}. Recent data show that TRAF6 induces the expression of muscle-specific E3 ubiquitin ligases and autophagy-related molecules in the skeletal muscle upon denervation and in LLC-bearing mice⁸. Moreover, TWEAK has been reported to induce the activation of both autophagy and proteasome in C2C12 myotubes⁵⁶. The results obtained in the present study show that TNF α directly stimulates autophagic degradation in both C2C12 and isolated murine satellite cells, suggesting another possible trigger of autophagy in cancer cachexia.

A relevant issue raised in the present work concerns the observation that TNF α exposure *in vitro*, as well as tumor growth *in vivo*, partially overcome the well known insulin/IGF-1 ability to down-regulate autophagy. These results could explain the virtual lack of effect of IGF-1/insulin-based strategies in preventing cancer-induced muscle wasting (^{57, 58}; present work). In this regard, a quite recent study reported that increased IGF-1 receptor engagement in the skeletal muscle results in rapid IRS-1 phosphorylation and proteasome-dependent degradation⁵⁹. On the contrary, IGF-1 overexpression effectively prevents glucocorticoid-induced muscle atrophy as well as the sarcopenia of aging^{22, 57}, although no information regarding the effect on autophagy in these model systems are actually available.

Conclusions

1 The results reported in the present study show that autophagy contributes to generate
2 muscle atrophy in cancer cachexia as well as in dexamethasone-treated animals, although the
3 underlying mechanisms in the two conditions are likely different. By contrast, sarcopenia of aging,
4 a slow but progressive physiological loss of muscle mass, does not seem to depend on autophagy.
5 These results further support the idea that the final event, e.g. muscle wasting, likely derives from
6 the combination of different mechanisms; in this regard, autophagy participates to variable extent
7 to the complicated network that leads to the loss of muscle mass. Finally, catabolic stimuli such as
8 the presence of a tumor or, more specifically, proinflammatory cytokines, cannot be fully
9 antagonized, at least in terms of autophagic degradation, by insulin, one of the most powerful
10 inhibitors of autophagy. These results are particularly intriguing, and could suggest that activation
11 of autophagy in these model systems is so enforced that cannot be circumvented by simple
12 inhibitor-based strategies.

13

1 **FIGURE LEGENDS**

2 Figure 1. Densitometric analysis and representative patterns of expression of autophagy-related
3 proteins in the GSN muscle of C and C26 bearers at 11 or 14 days after tumor transplantation (A).
4 Densitometric analysis and representative patterns of expression of LC3B protein in the
5 gastrocnemius of C and C26 hosts at day 11 of tumor growth either untreated or treated with
6 colchicine (Col; B). Data (mean \pm SD) are expressed as percentages of C. Significance of the
7 differences: * $p < 0.05$ vs C, ** $p < 0.01$ vs C, *** $p < 0.001$ vs C, ## $p < 0.01$ vs Col, \$\$\$ $p < 0.01$ vs C26.

8 Figure 2. Expression of genes involved in atrophy (A) and cathepsin B+L activity (B) in the GSN of C
9 and C26 hosts at day 14 and 11 of tumor growth. Data (mean \pm SD) are percentages of C. As for
10 gene expression results are expressed as mean \pm SEM. Significance of the differences vs C:
11 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

12 Figure 3. Gastrocnemius muscle mass and expression of autophagy-related proteins in GSN of AH-
13 130 (A, B) or LLC tumor-bearing animals (C, D). Data (mean \pm SD) are expressed as percentages of
14 C. Significance of the differences vs C: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

15 Figure 4. Loss of body weight and skeletal muscle mass in dexamethasone-treated mice (A; GSN =
16 gastrocnemius, TIB = tibialis). Densitometric analysis and representative patterns of expression of
17 autophagy-related proteins in the GSN muscle of C and DEXA-treated mice (B). Expression of genes
18 involved in atrophy (C) and cathepsin B+L activity (D) in the GSN of C and DEXA-treated mice. Data
19 (mean \pm SD), are percentages of C. As for gene expression results are expressed as mean \pm SEM.
20 Significance of the differences vs C: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

21 Figure 5. Densitometric analysis and representative patterns of expression of autophagy-related
22 proteins in the gastrocnemius (GSN) of 3 and 22 month old mice (A) and 6 or 24 months old rats
23 (B), fed ad libitum (AL) or with 40% calorie restriction (CR). Data (mean \pm SD), are percentages of
24 C. Significance of the differences vs C: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; vs AL24: \$ $p < 0.05$.

25 Figure 6. Loss of body weight and skeletal muscle mass in insulin-treated (1,5 IU/kg b.w.) animals
26 (A; GSN = gastrocnemius, TIB = tibialis). Densitometric analysis and representative patterns of
27 expression of autophagy-related proteins in the GSN muscle of C and C26 hosts, either treated or
28 untreated with insulin (B). Expression of genes involved in atrophy in the GSN of C, C26 and INS-
29 treated C26 hosts (C). Data (mean \pm SD), are percentages of C. As for gene expression, results are

1 expressed as mean \pm SEM. Significance of the differences vs C: * $p < 0.05$, *** $p < 0.001$; vs C26
2 $\$p < 0.05$, $\$\$p < 0.01$.

3 Figure 7. C2C12 myotubes (2 day differentiation) treated for 48 h with $\text{TNF}\alpha$, in the presence or in
4 the absence of INS. Densitometric analysis and representative patterns of expression of autophagy
5 related proteins and of phospho-Akt (A). Data (mean \pm SD; $n=3$) are expressed as percentages of
6 controls. Significance of the differences: * $p < 0.05$ vs C, ** $p < 0.01$ vs C; $\$p < 0.05$ vs $\text{TNF}\alpha$. (B) p62
7 and LC3B immunostaining (green: p62 or LC3B-II; blue: HOECHST).

8 Figure 8. Densitometric analysis of expression of autophagy related proteins in C2C12 cells
9 cultured in the presence or in the absence of bafilomycin A1 (BAF; A) and/or insulin (B). Data
10 (mean \pm SD; $n=3$) are expressed as percentages of controls. Significance of the differences:
11 * $p < 0.05$ vs C, ** $p < 0.01$ vs C; # $p < 0.05$ vs BAF; $\$p < 0.05$ vs BAF INS.

12 **SUPPLEMENTAL FIGURE LEGENDS**

13 Figure S1. Loss of body (A) and skeletal muscle weight (B; GSN = gastrocnemius, TIB = tibialis) in
14 Controls and C26 hosts (C=6, C26=8) at day 11 and 14 of tumor growth.

15 Figure S2. Densitometric analysis and representative patterns of expression of autophagy-related
16 proteins in the liver of C and C26 hosts (A), or C, C26 bearers and INS-treated C26 hosts (B) at day
17 14 of tumor growth. Data (mean \pm SD) are expressed as percentages of C. Significance of the
18 differences vs C: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; vs C26: $\$\$p < 0.01$.

19 Figure S3. Daily food intake (A) and insulin tolerance test (B) in C, C26 and INS-treated C26 hosts.
20 Correlation between glycemia and Δ i.b.w. in C26 hosts, either untreated ($r=0.693$) or INS-treated
21 ($r=0.939$; C).

22 Figure S4. (A) LC3B immunostaining in primary cultures of tibialis-derived satellite cells untreated
23 or treated for 48h with $\text{TNF}\alpha$ (100 ng/ml; green = LC3B, blue = HOECHST). (B) Densitometric
24 analysis and representative patterns of expression of LC3B-I and LC3B-II in C2C12 myotubes
25 exposed to 5 ng/ml $\text{TNF}\alpha$. Significance of the differences: * $p < 0.05$ vs C, ** $p < 0.01$ vs C, $\$p < 0.05$ vs
26 $\text{TNF}\alpha$.

1 Figure S5. Densitometric analysis and representative patterns of expression of LC3B-I and LC3B-II
2 in the muscle of C and TNF α -treated mice, in the presence or in the absence of colchicine
3 administration (see Materials and methods). Significance of the differences vs C: *p<0.05,
4 **p<0.01.

5 Figure S6. (A)LC3B immunostaining in C2C12 myotubes (2 day differentiation) untreated or
6 treated for 48 h with TNF α (100ng/ml), in the presence or in the absence of IGF1 (50 ng/ml) or
7 anti-IGF1 antibody (a-IGF1 = 2ug/ml). (B) Densitometric analysis and representative patterns of
8 expression of LC3B and phospho-Akt. Data (mean \pm SD; n=3) are expressed as percentages of C .
9 Significance of the differences vs C: *p<0.05, **p<0.01.

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Figure 1

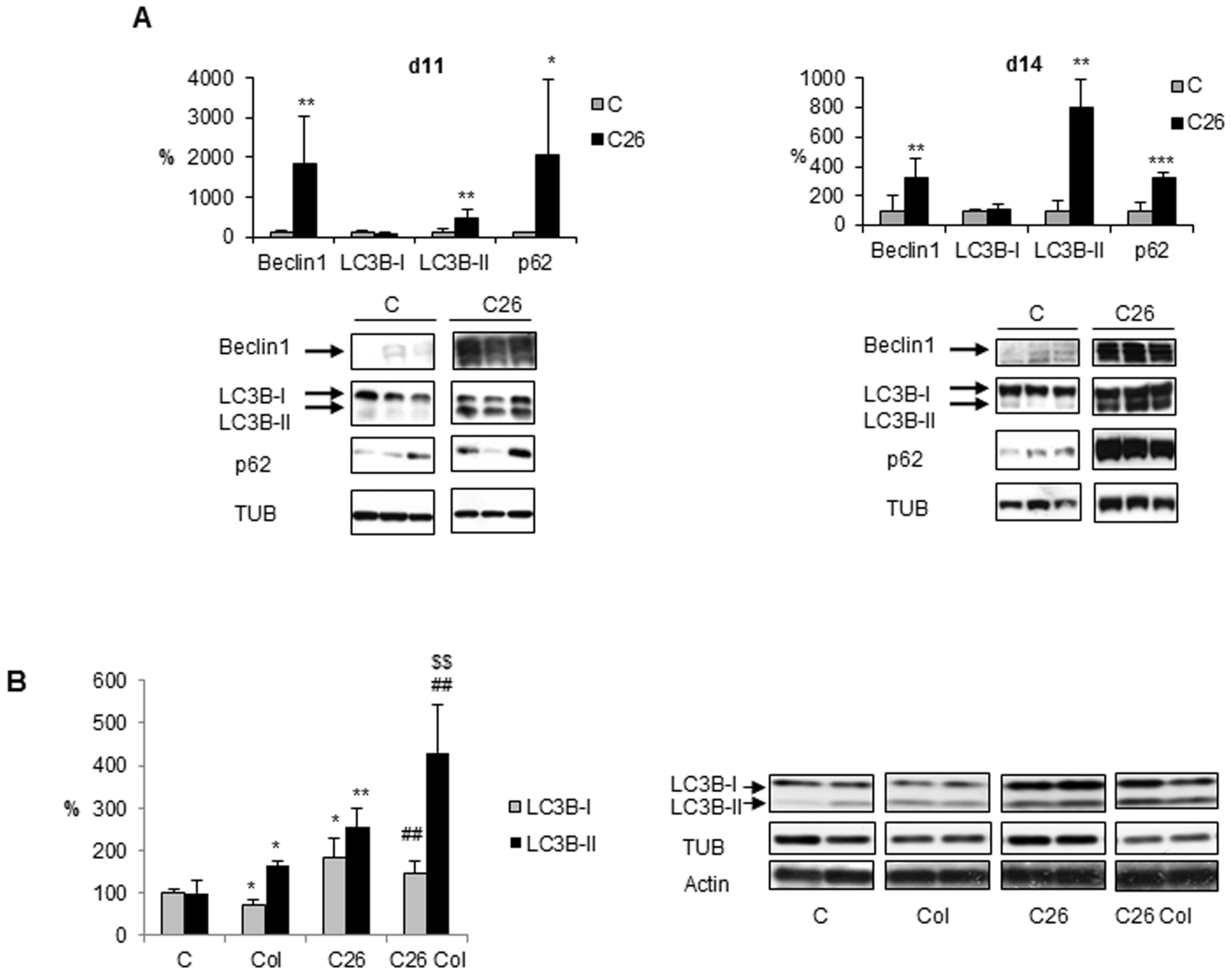
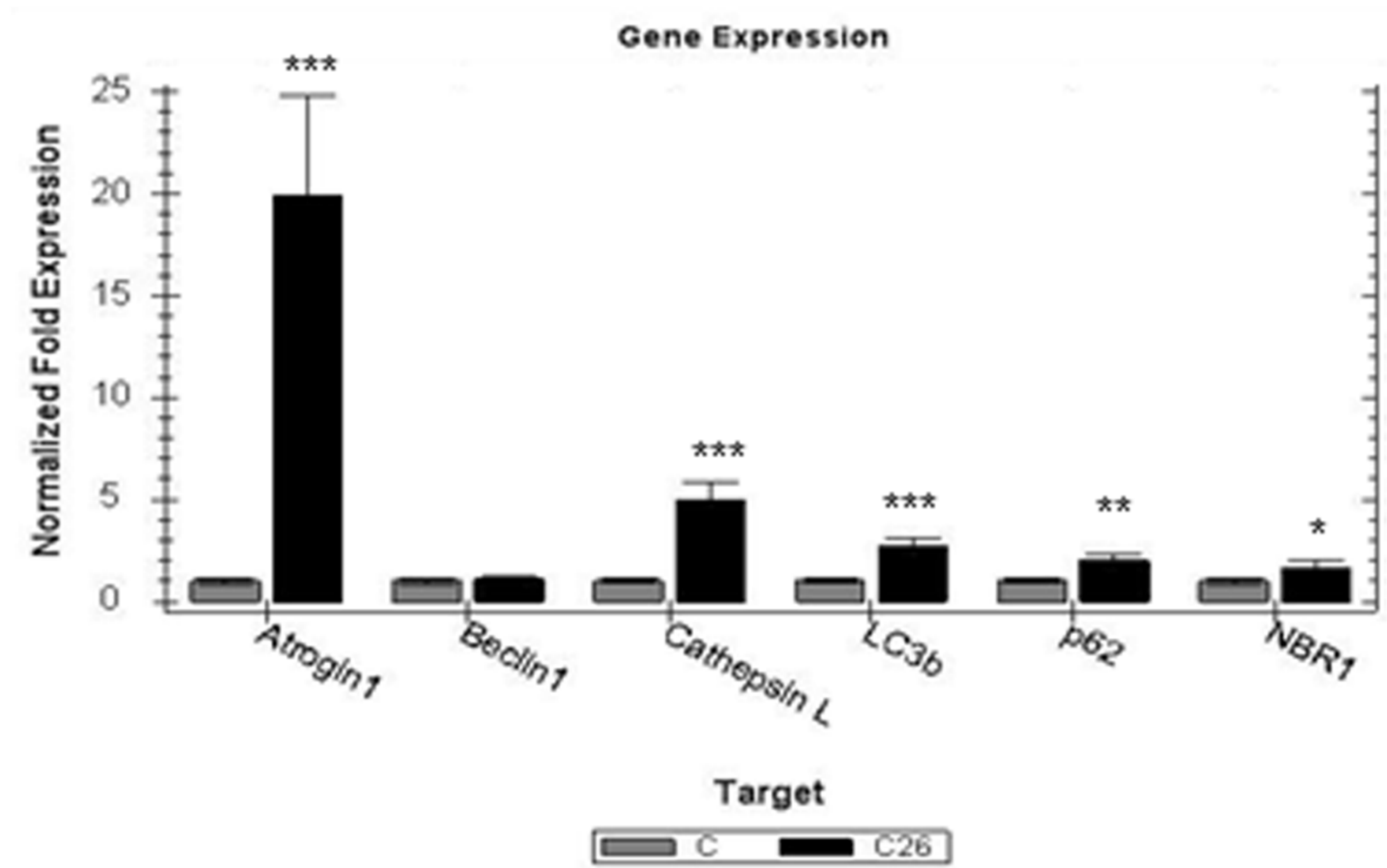


Figure 2

A



B

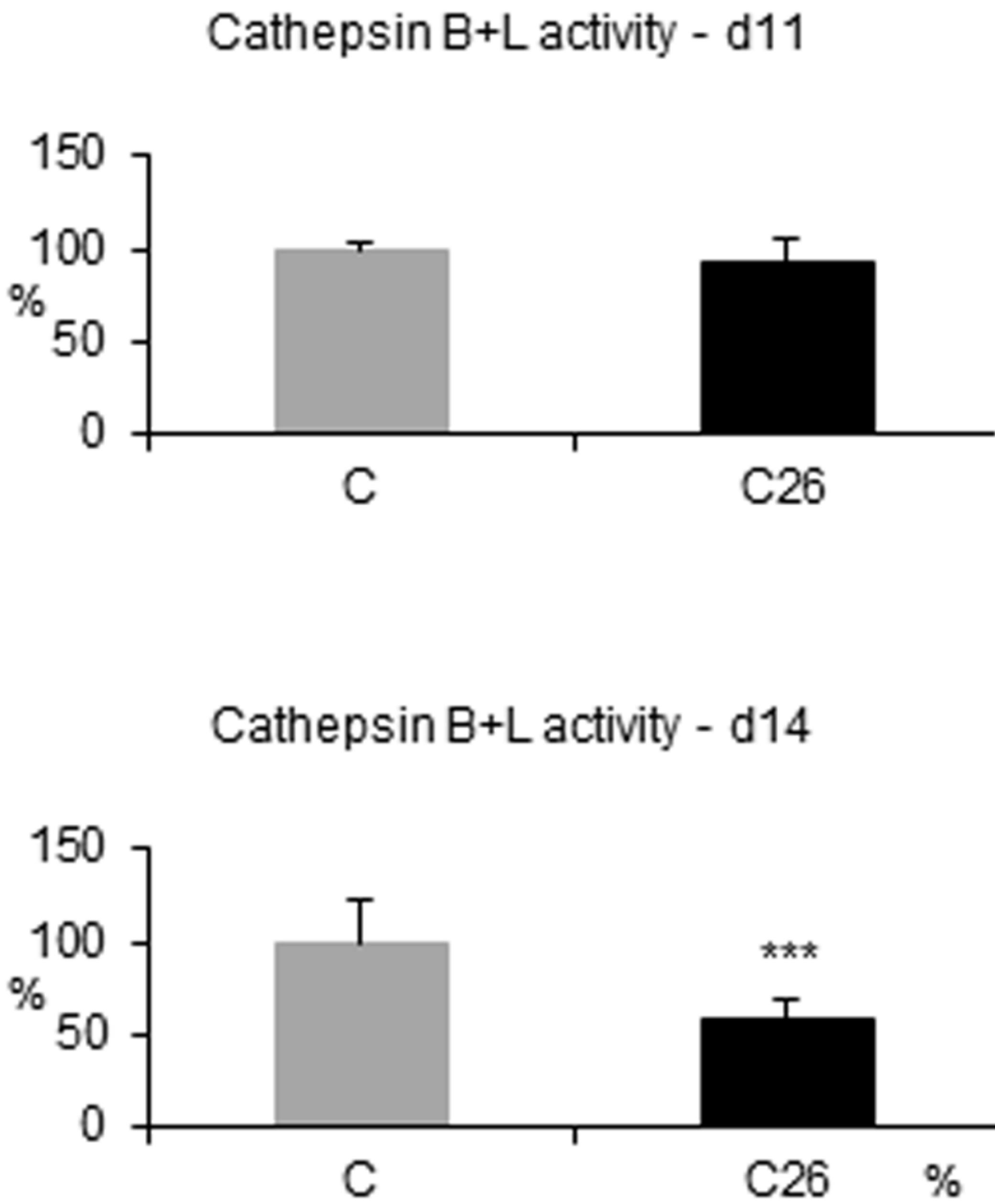


Figure 3

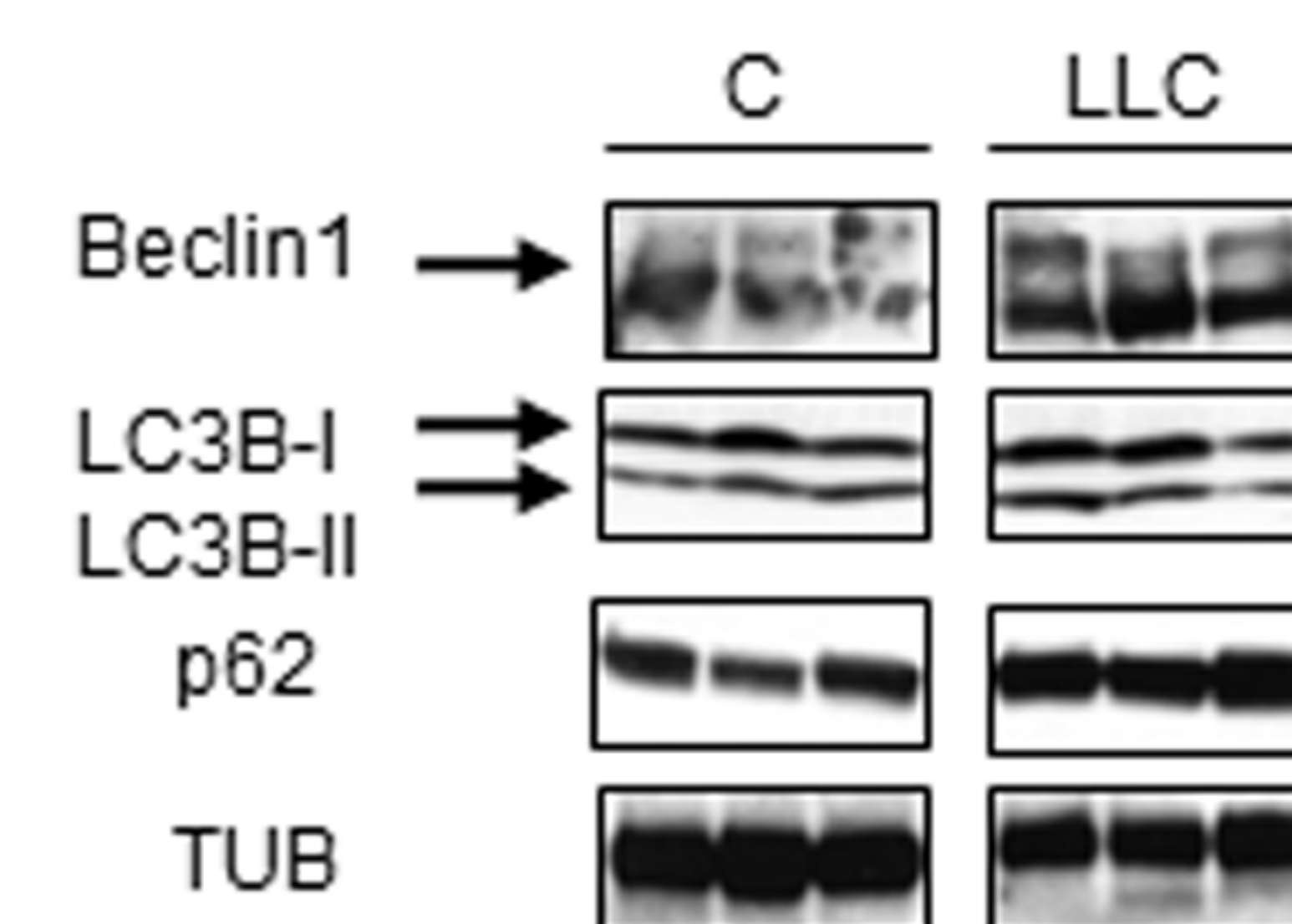
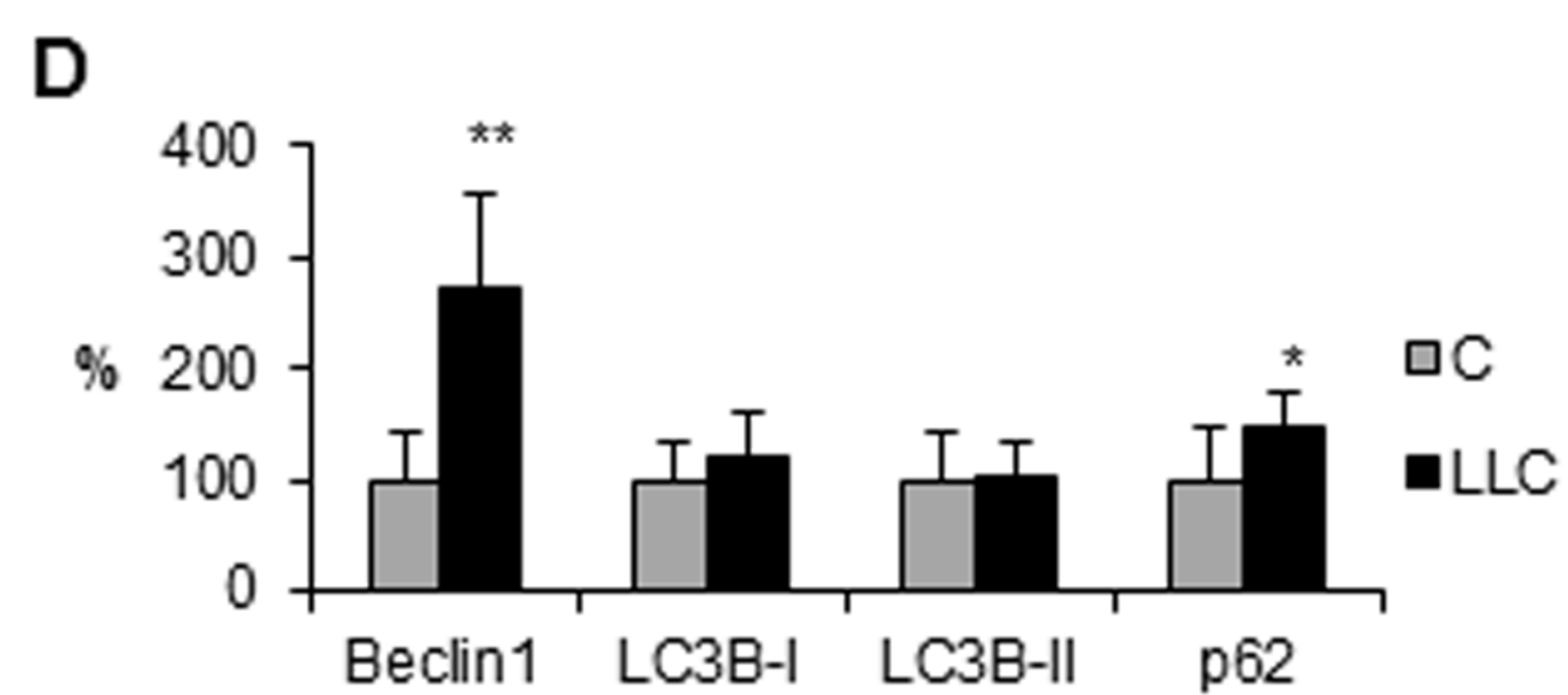
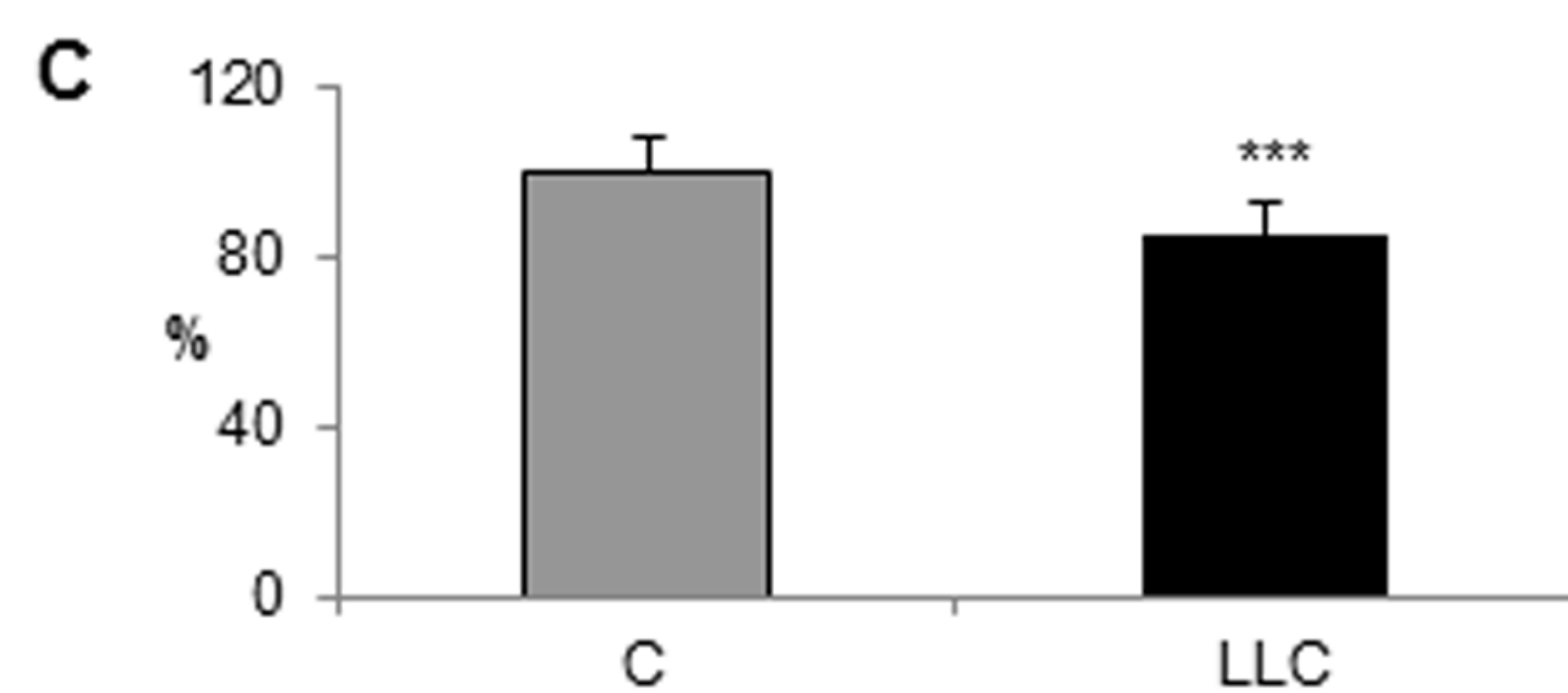
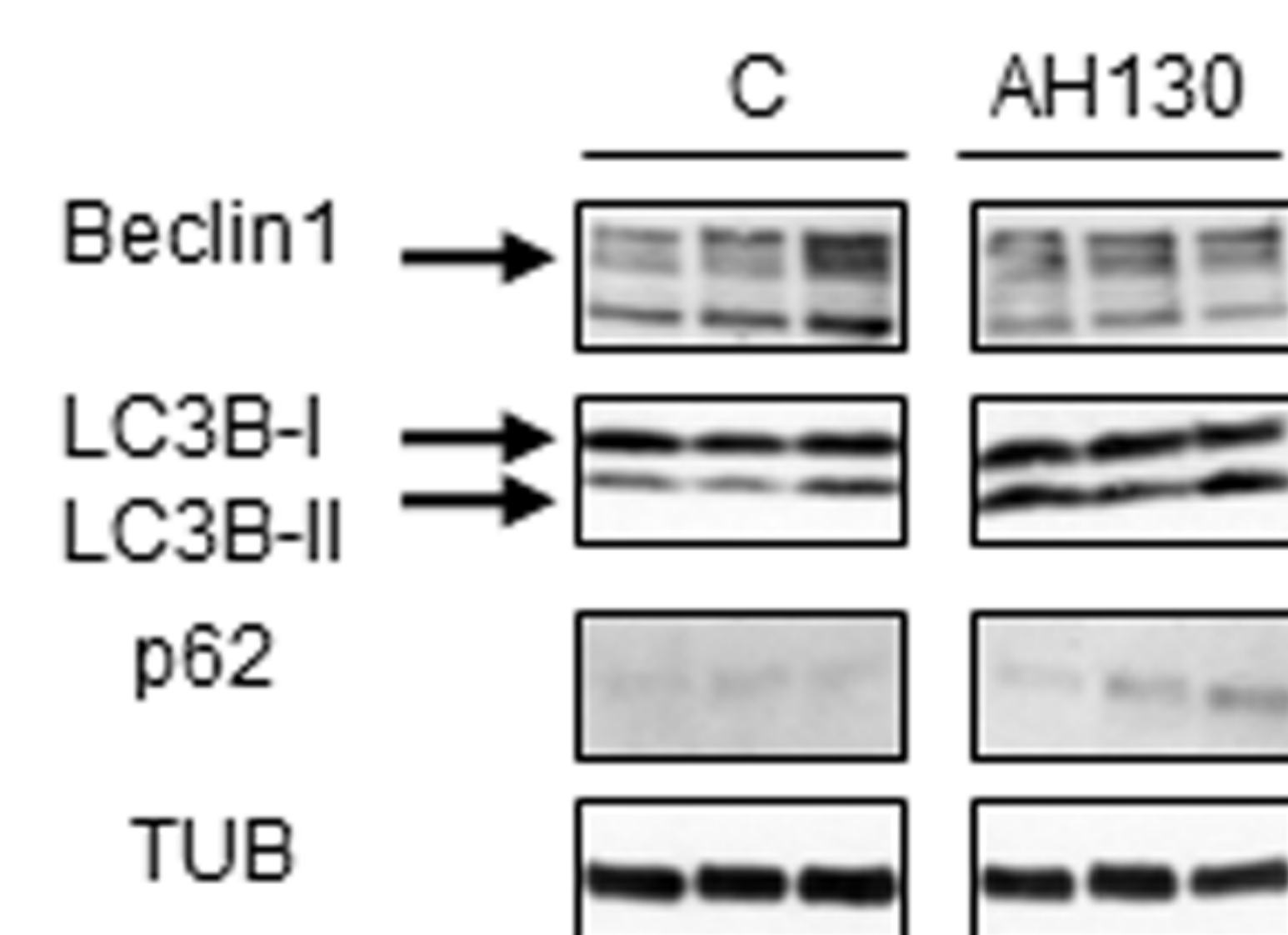
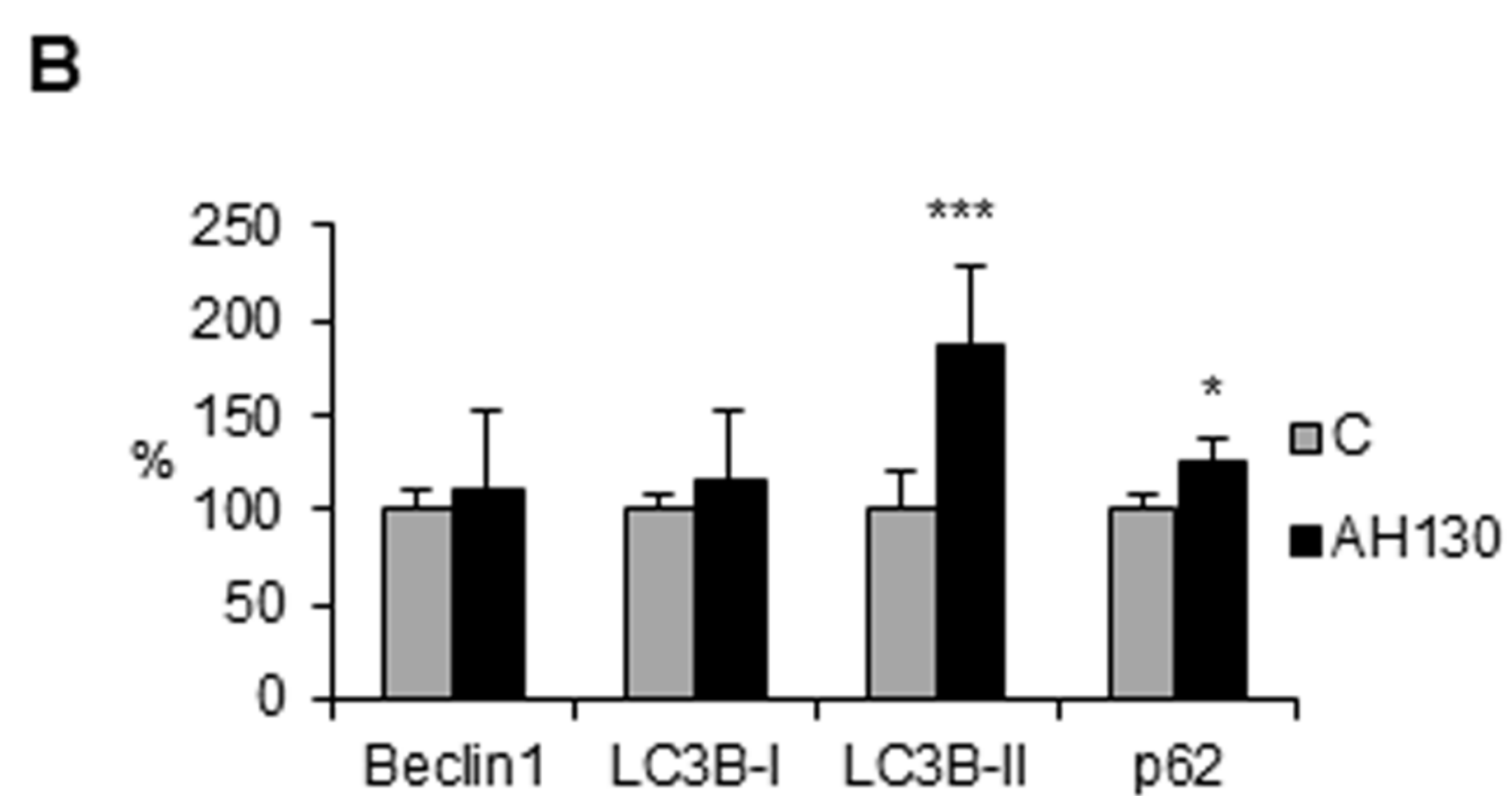
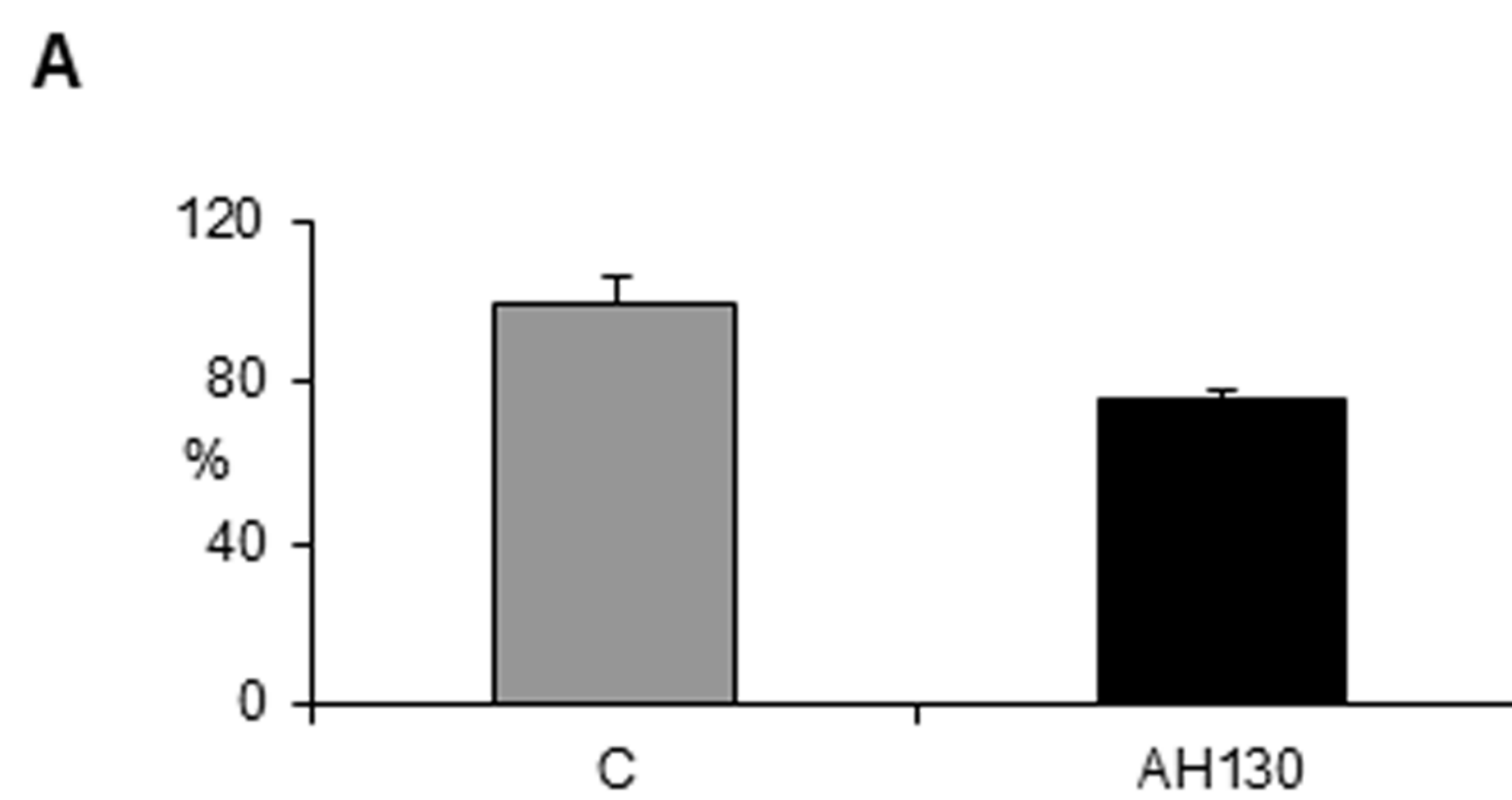
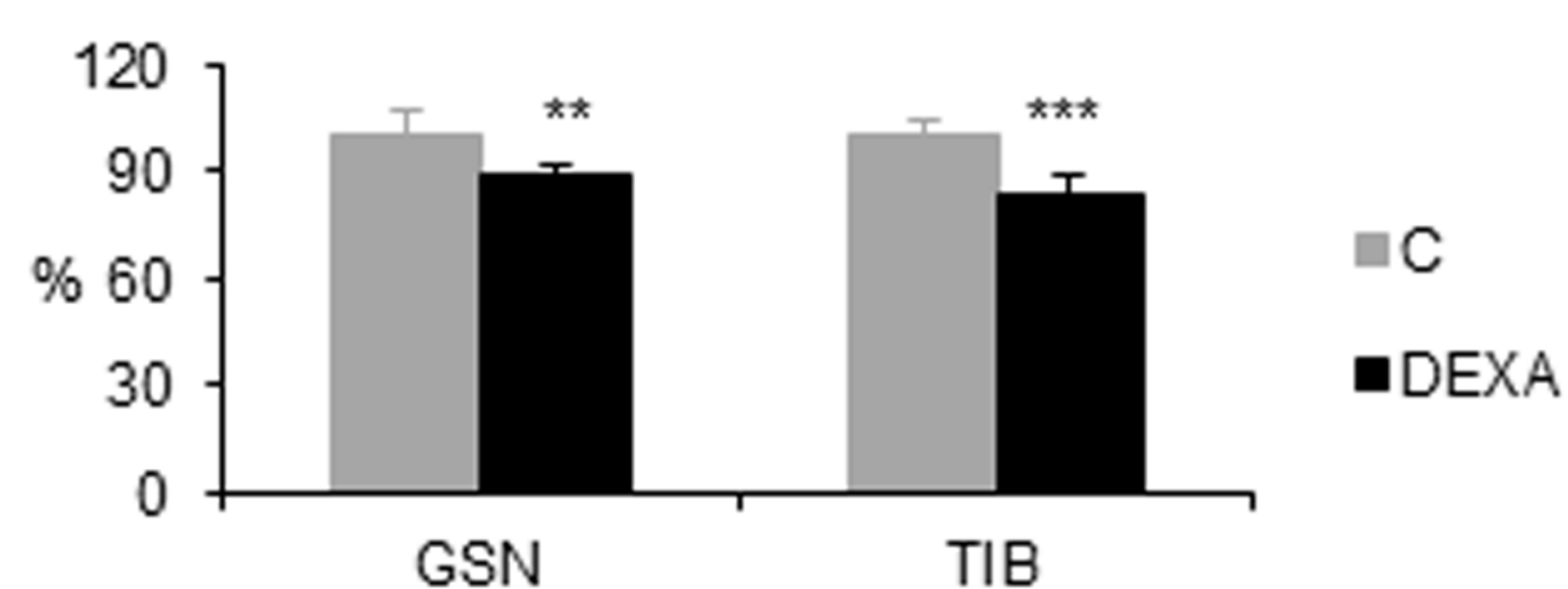
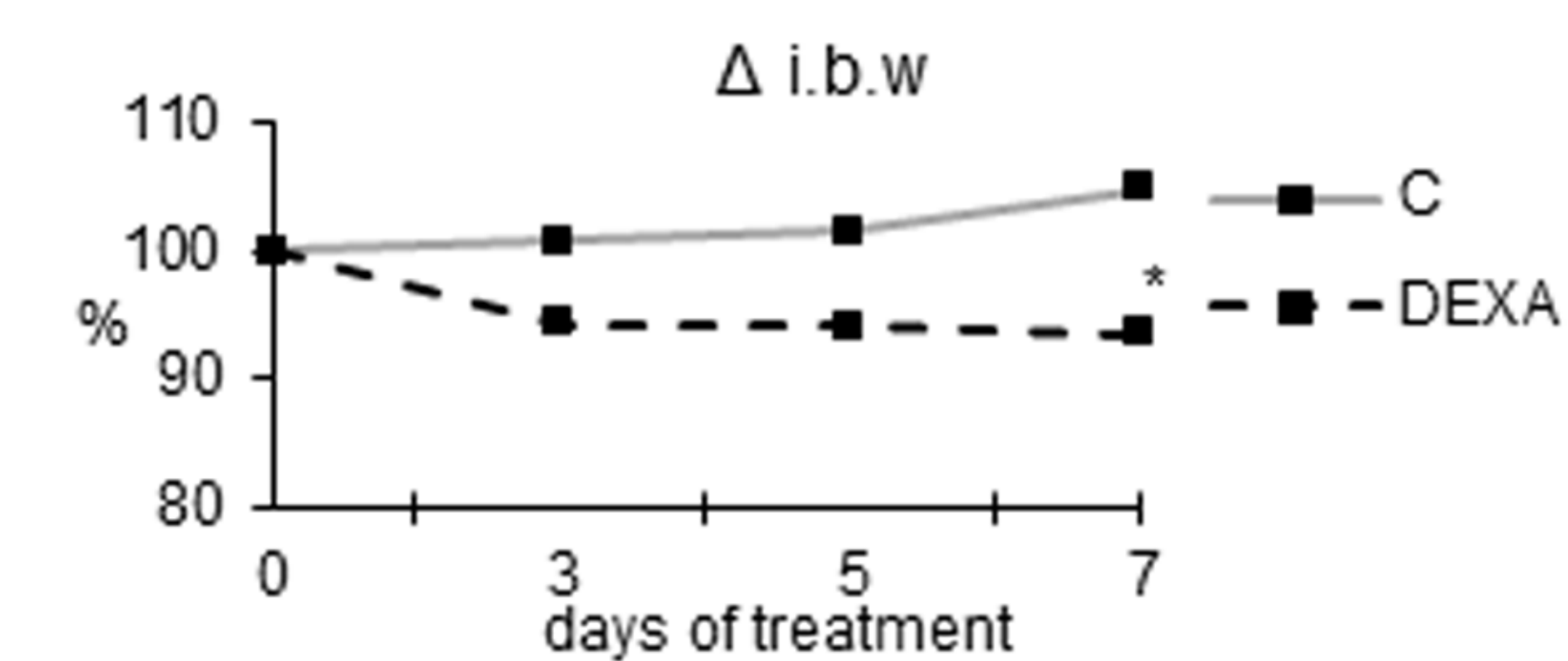
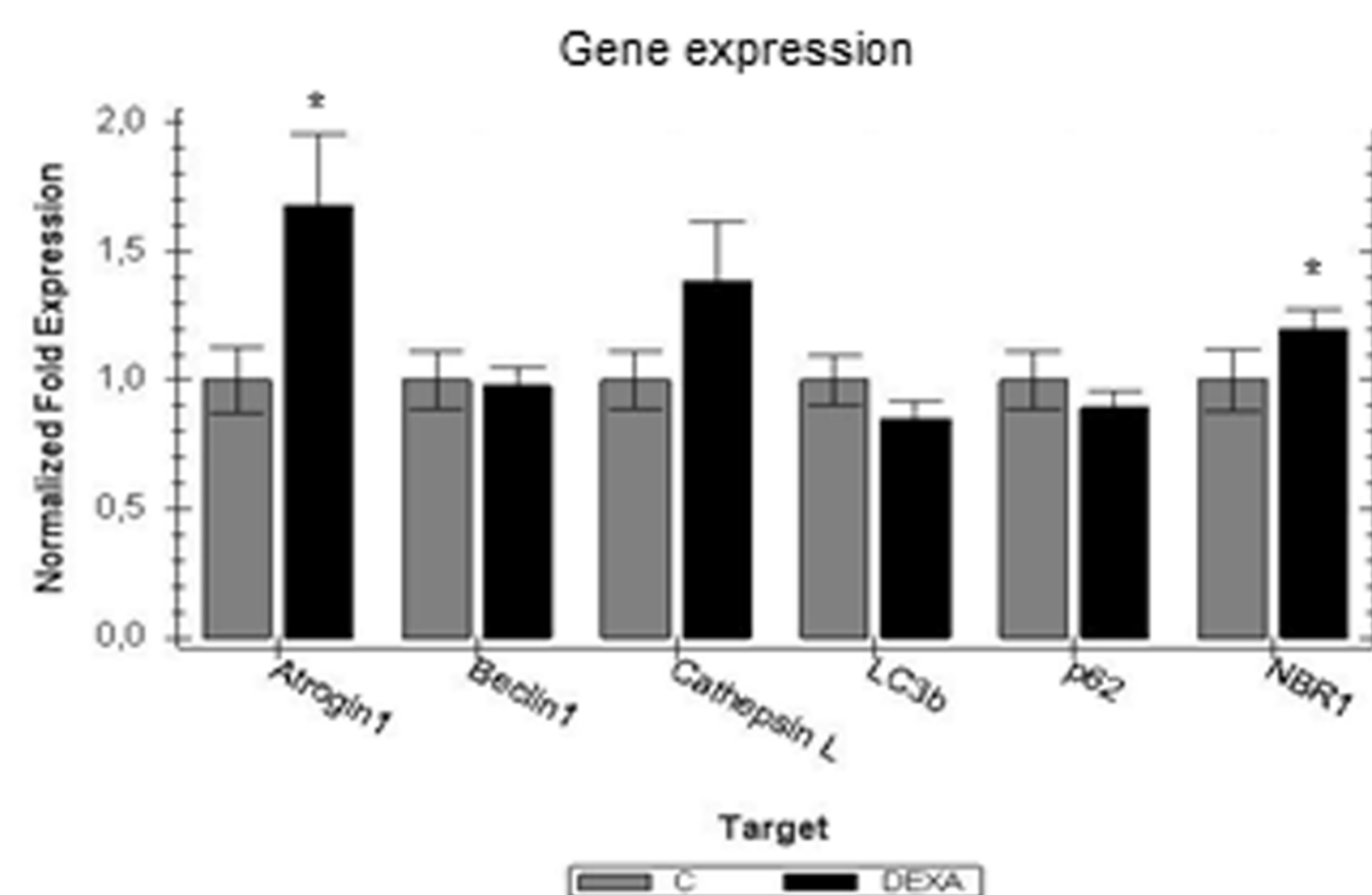


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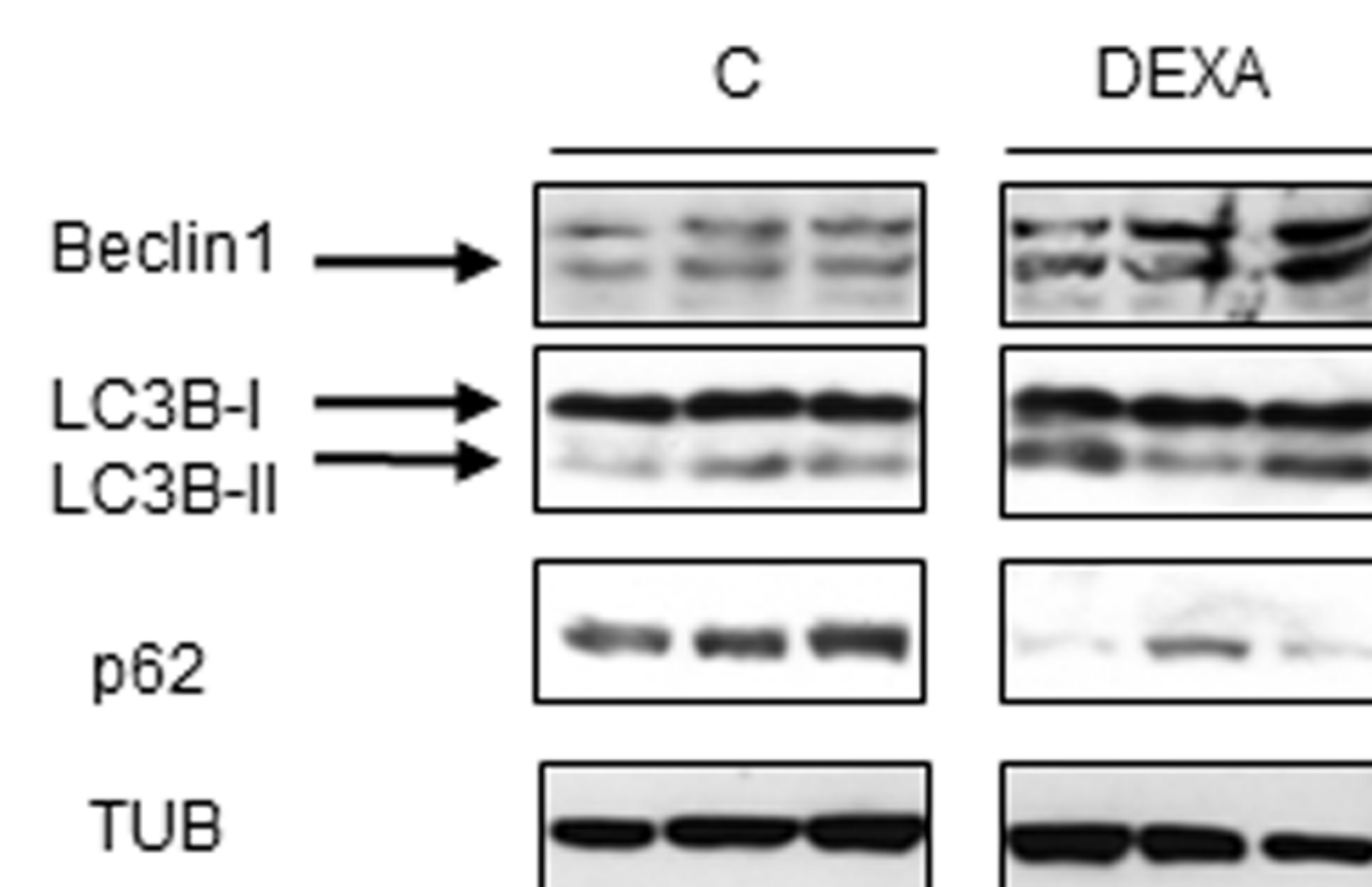
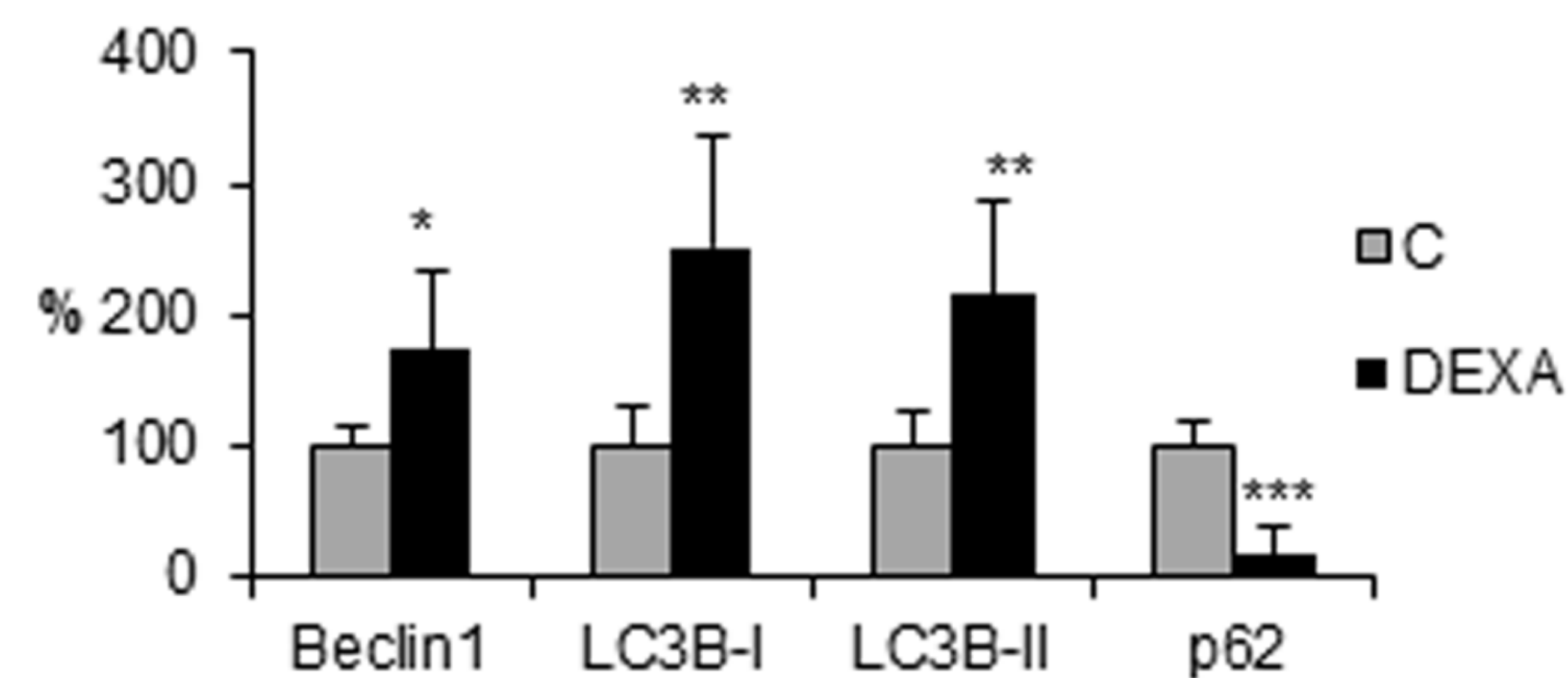
A



C



B



D

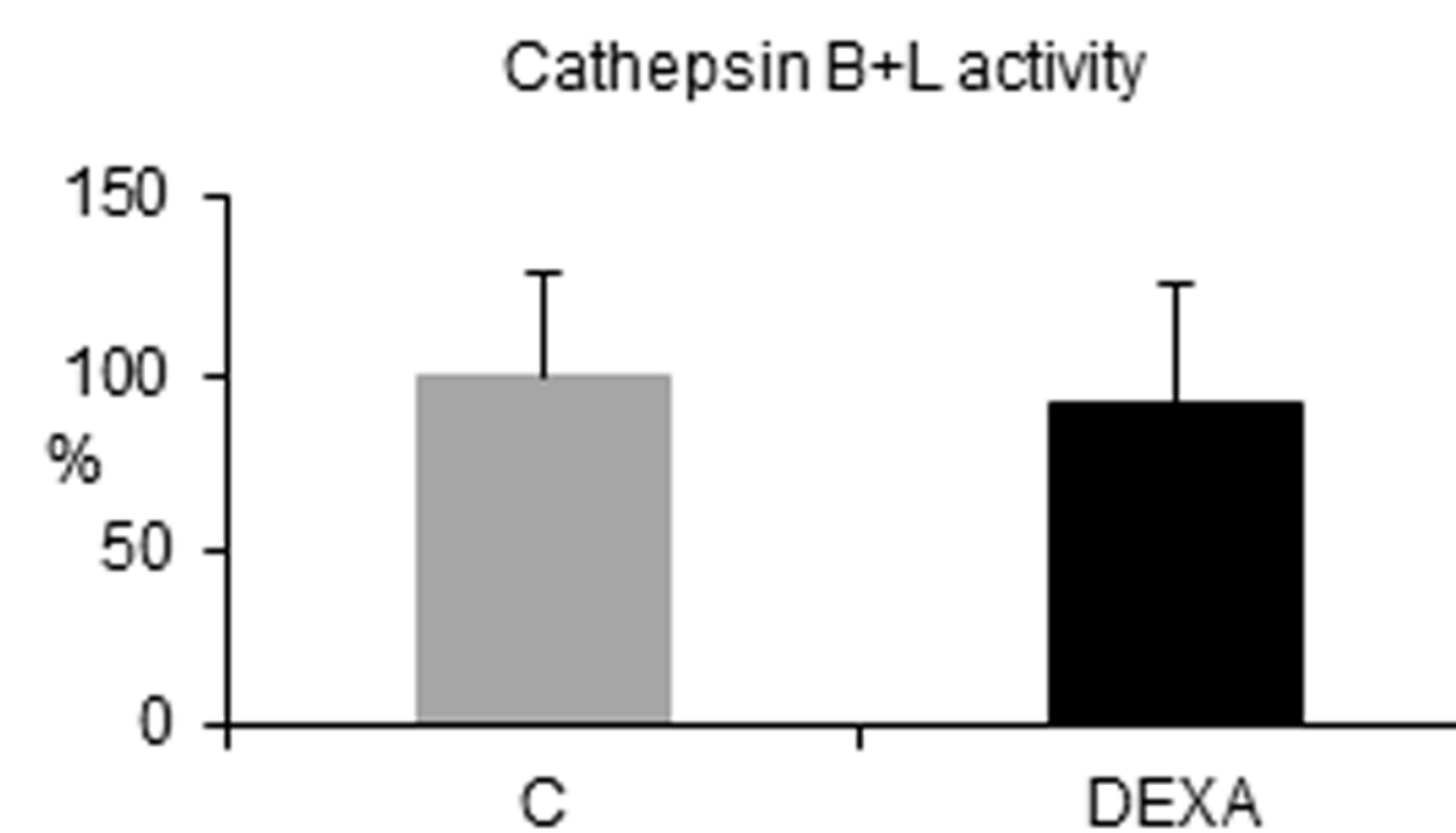
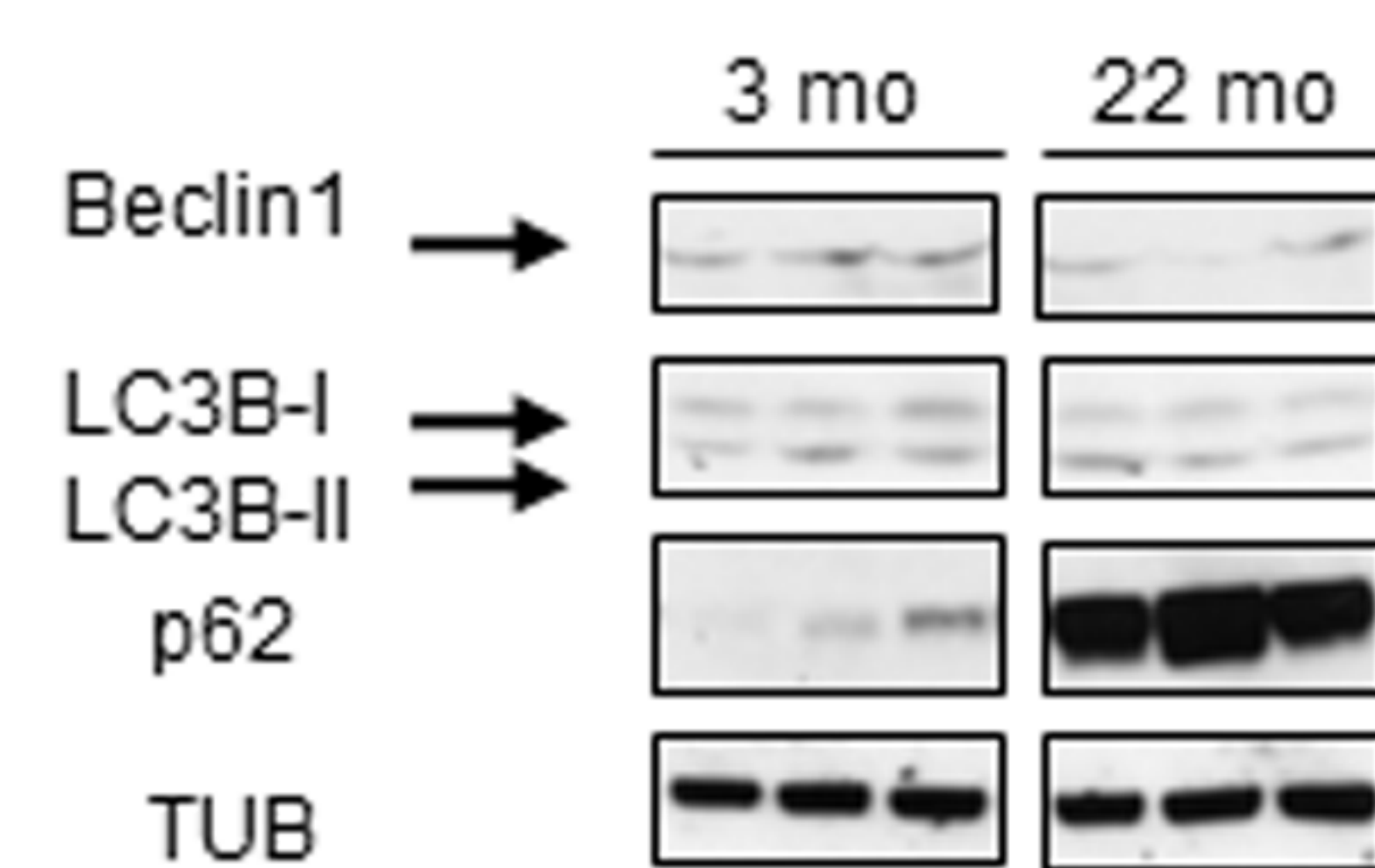
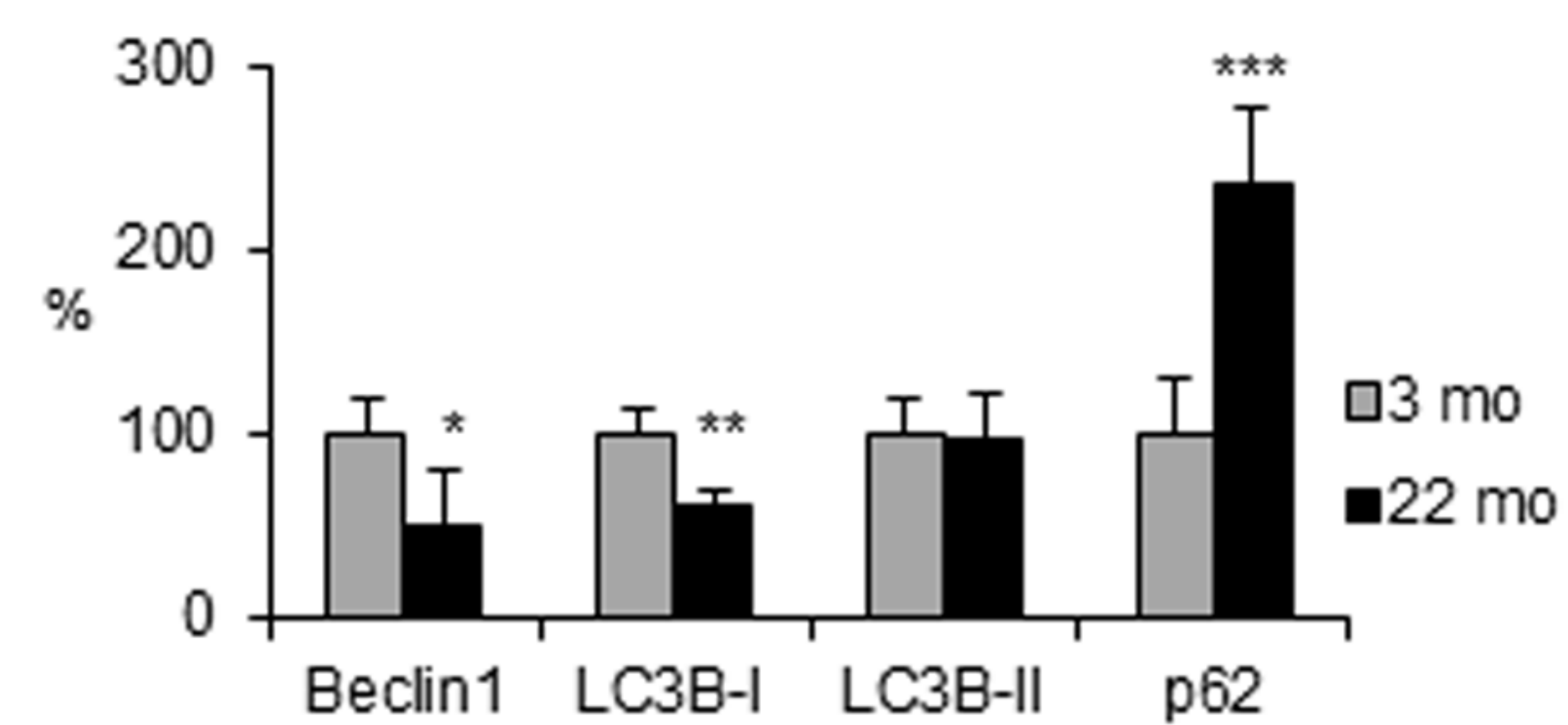


Figure 5

A



B

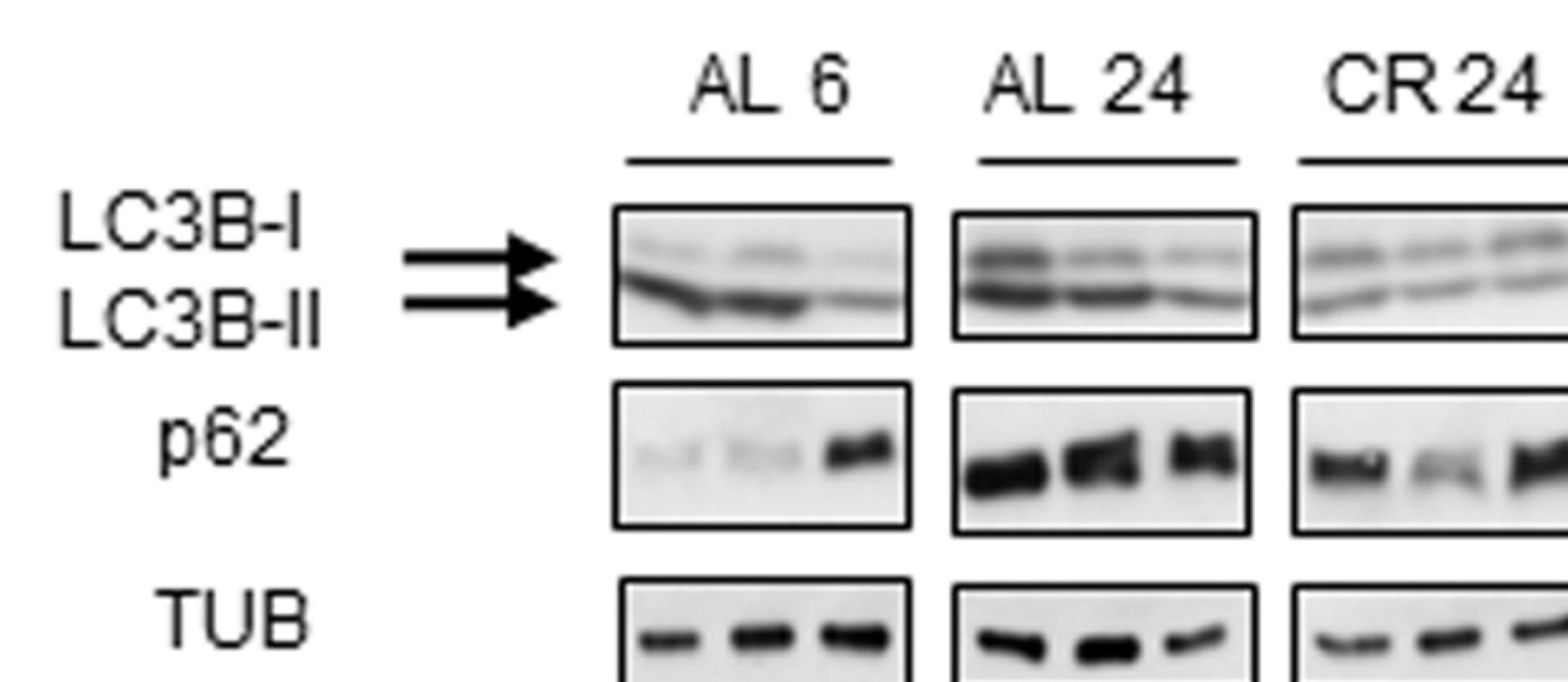
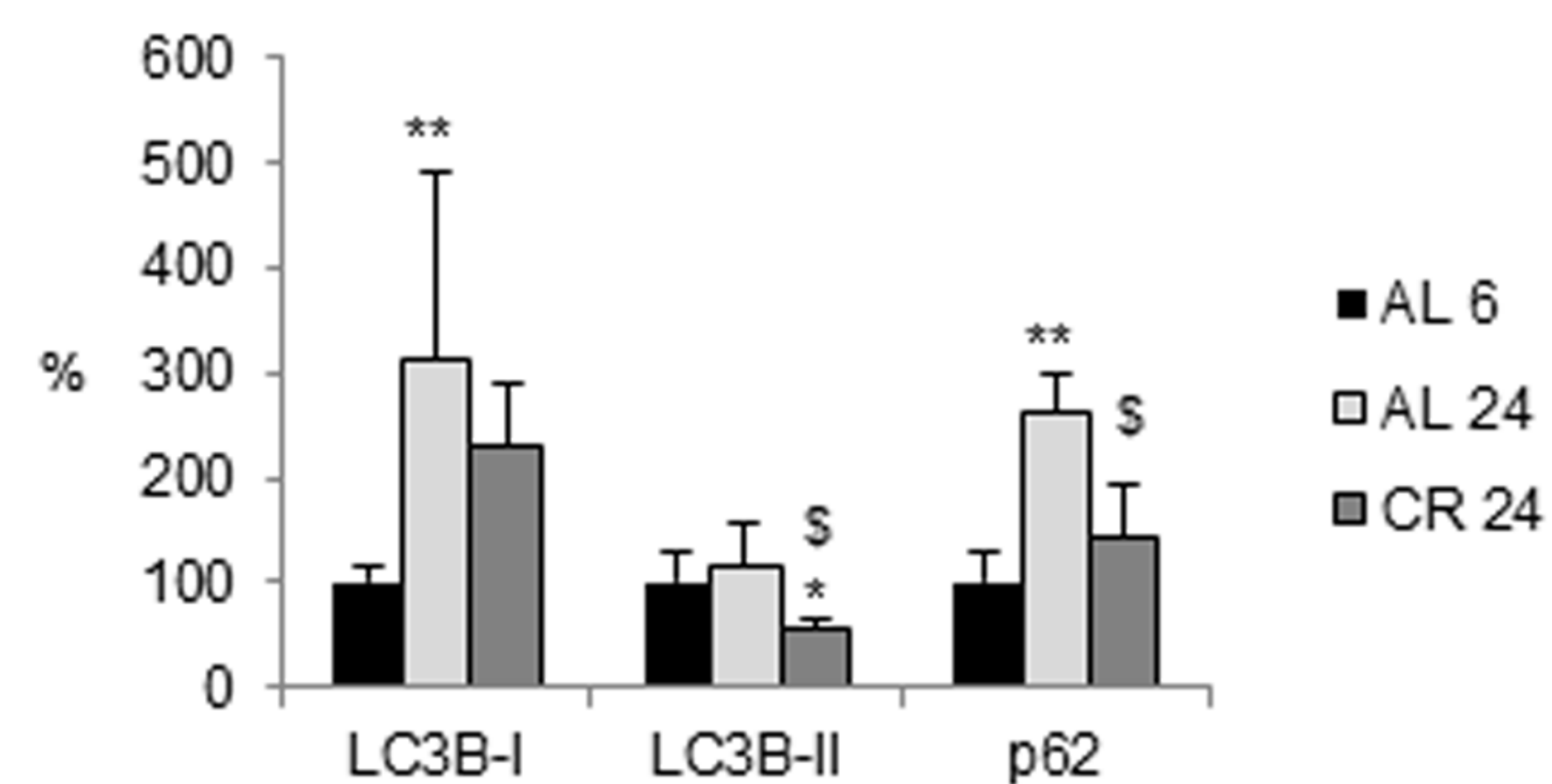


Figure 6

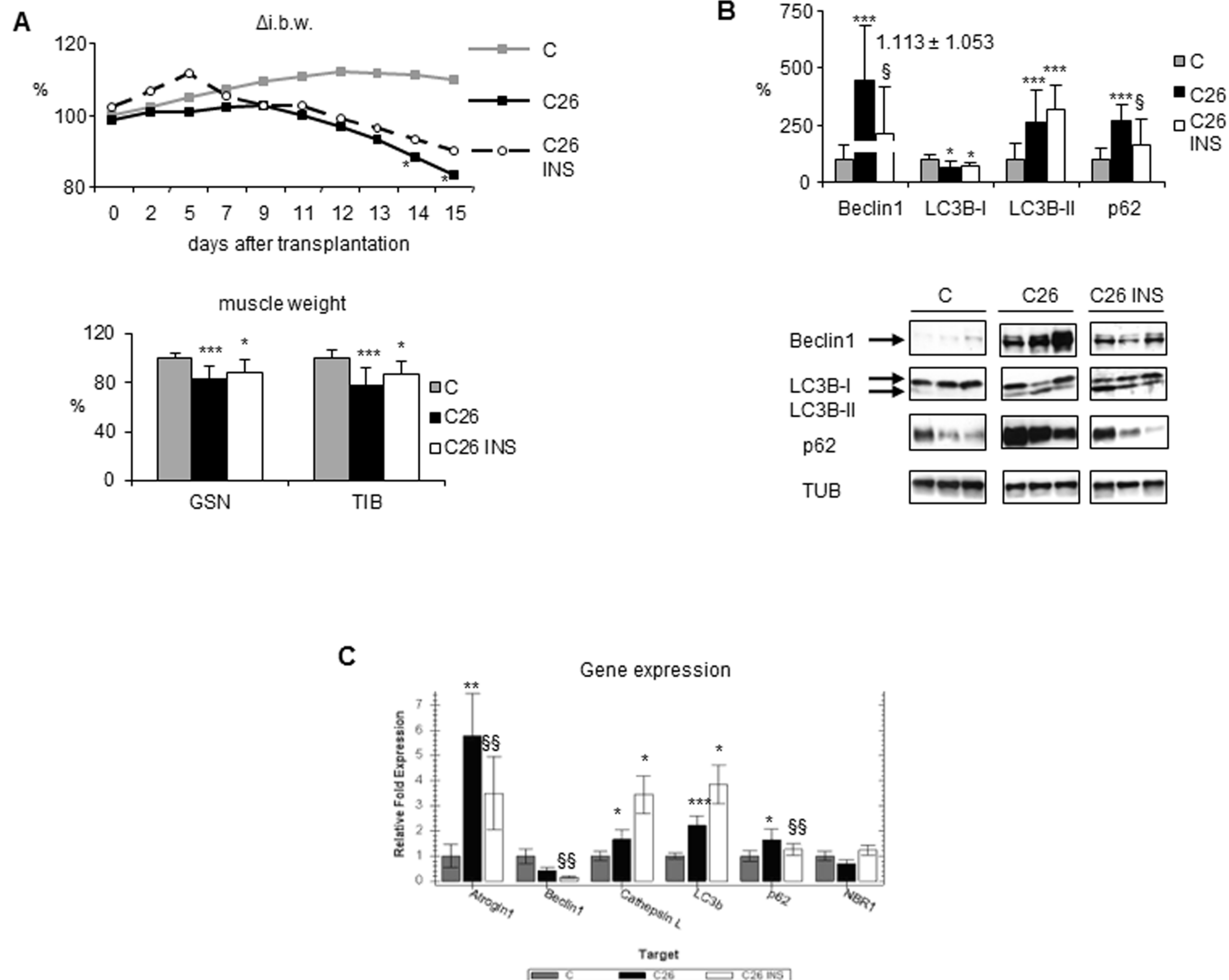
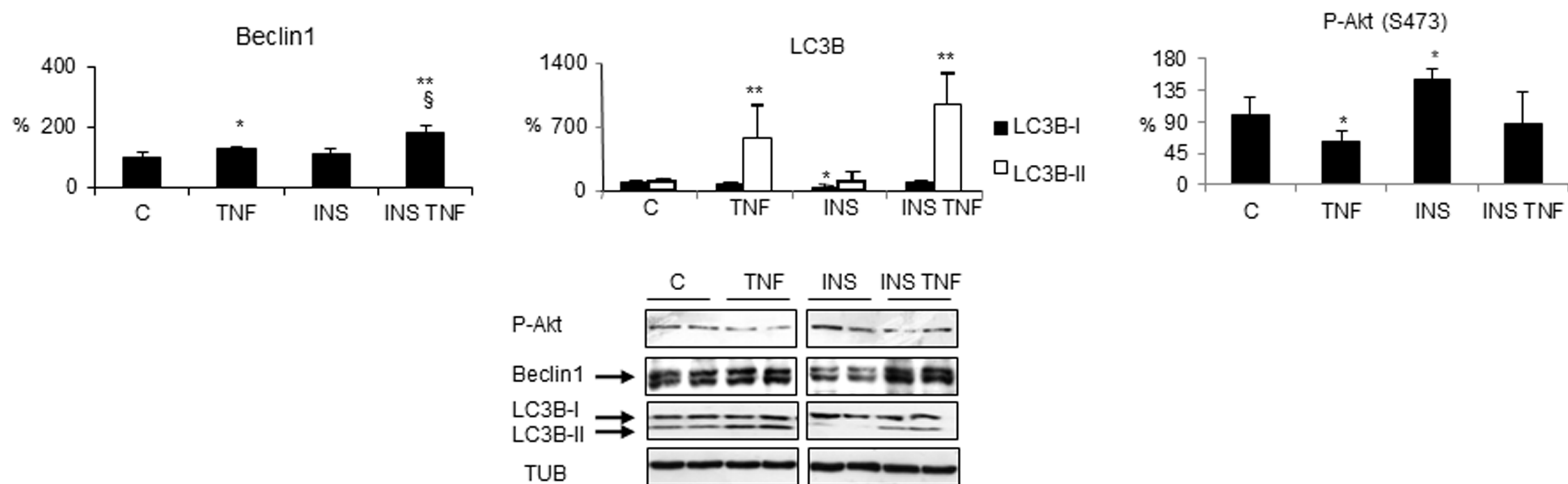


Figure 7

A



B

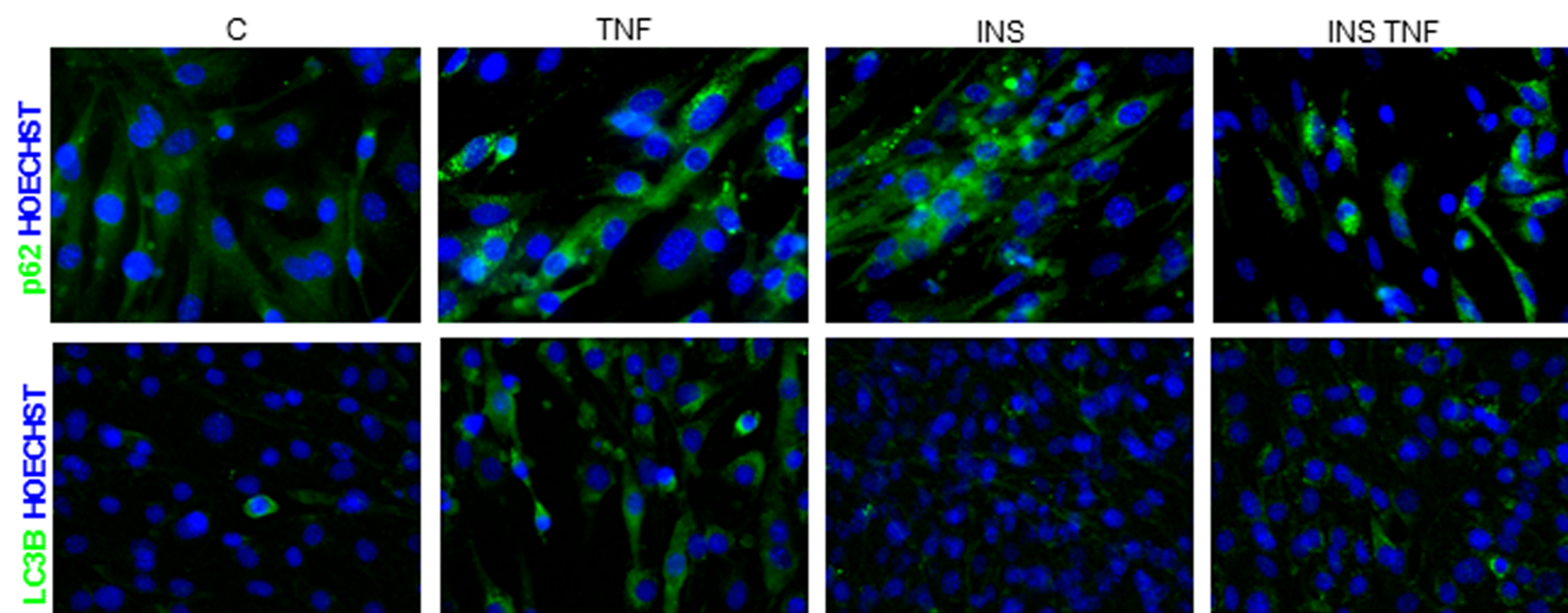


Figure 8

